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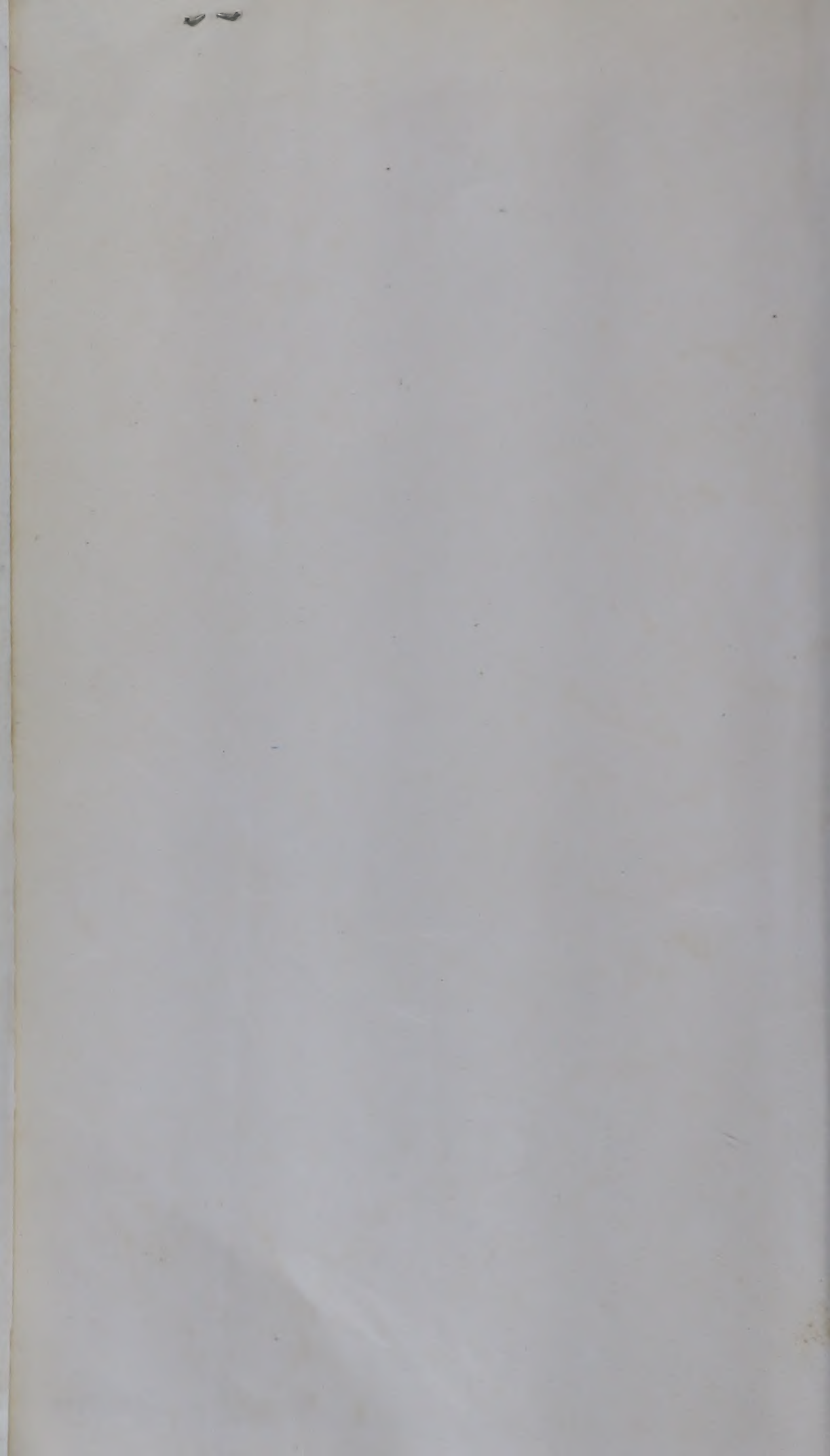


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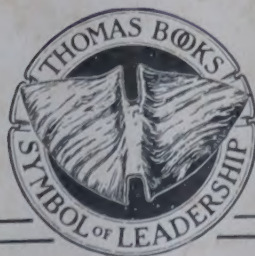
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AMINO ACIDS and PROTEINS

THEORY • METHODS • APPLICATION

Compiled and Edited by
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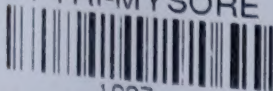
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PREFACE

IMPORTANT GOALS of the researches on proteins are to determine their chemical composition and structure, to develop improved methods of isolation, to establish their purity, to measure their size and shape, to explore their physical and chemical characteristics, to develop precise methods for the analysis of their component units and products, and to evaluate the functions and properties of biologically important proteins, and of protein derivatives.

This book is devoted to the presentation of the methods and results of the main channels of effort by which these goals are being achieved or, at least, approached. It is intended to acquaint students and investigators with the more general methods employed in the study of the proteins and to give a summary of the current knowledge and advances of the subject matter. Topics requiring advanced mathematical treatment have not been included.

This book is a cooperative effort by a group of men well known for their contributions to the special subject matter of the book on which they have written.

May I also point out that the present work stems from the deep and abiding interest in the study of the proteins in the belief that it is a key to an understanding of the phenomenon of life which has been a tradition of the Biochemistry laboratory of our University from its inception. This interest was kindled by the pioneer efforts of such notables as Jaques Loeb and T. Brailsford Robertson. The inspiration received from these men was passed on to and maintained in his pupils by the late Carl L. A. Schmidt, among whom I have the honor to be numbered. The publication of books on proteins from the Biochemistry laboratory is traditional. I hope the present volume is a worthy successor to its predecessors.

Lastly, it gives me great pleasure to acknowledge the valuable assistance of Dr. Theodore Winnick in the editorial work of preparing many of the chapters for publication.

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AMINO ACIDS and PROTEINS

THEORY • METHODS • APPLICATION

Chapter I

PROPERTIES OF AMINO ACIDS

E. E. HOWE, PH.D.

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I. NOMENCLATURE

THE NOMENCLATURE of the amino acids is at present emerging from a state of confusion. Originally, upon the discovery and isolation of an amino acid, its direction of rotation was determined and the new compound was prefixed d- or l- depending upon whether it was dextro- or levo-rotatory. As the configurational relationship of the amino acids was gradually elucidated it developed that all of the natural acids isolated from proteins, very probably, have the same configuration about the α -carbon atom and that this configuration is identical with that of l(+)-lactic acid. Accordingly, this relationship was expressed by the prefix l- regardless of direction of rotation and the proteinogenic amino acids became known as the l-series.

In order to prevent confusion with the old system, the direction of rotation was indicated by + or - signs in parentheses so that the glutamic acid found in protein hydrolysates, for example, known as d-glutamic acid by the old system became l(+)-glutamic acid by the new. Since the rotation of an amino acid varies in acid and alkaline solution and with temperature, it was necessary before the compound could be properly named to define the conditions under which the rotation was determined. Unfortunately there is some difference of opinion concerning these conditions. Dunn (78) has suggested that the sign of the prefix be determined by the direction of rotation of the free amino acid in water at 25°. On the other hand, since the observed rotation is often more consistent when determined in the presence of an excess of acid or base, Clarke (53) advocated that the sign of rotation of an amino acid be determined in the presence of four or more equivalents of acid.

In 1936 the situation was further complicated when upon the

isolation and clarification of the structure of natural threonine (167) it was designated as d(−)-threonine because of its spatial relationship to d(−)-threose. The spatial arrangement of the groups about the α -carbon atom of this compound is identical with that of the other natural amino acids and it therefore belongs to the l-series.

Until recently no group or organization had taken a definite stand and adopted a well defined system of nomenclature with the consequence that both systems are still appearing in the literature.

In the opinion of many, neither system is desirable. If the old system is used the reader must know beforehand the direction of rotation of the amino acid before he can identify it with respect to series or family which, after all, is the only thing he wishes to do. The new system often becomes confused with the old, is unnecessarily long, awkward, and makes no definite provision for the naming of derivatives, i.e., it does not indicate whether they should be given the sign of rotation of their parent substances or should be named according to their own sign of rotation.

Recently, under the leadership of Dr. H. B. Vickery, one of the the editors of the *Journal of Biological Chemistry*, the American Chemical Society's Committee on Nomenclature has approved a set of rules for the clarification of amino acid nomenclature (12, 234). These rules, although necessarily detailed to cover all contingencies, are simple and logical, consisting, in essence, of the use of the small capital letters D and L to indicate the two series or families of the amino acids and their derivatives. This method of nomenclature is in keeping with the system adopted by the American Chemical Society for the carbohydrate series and will be used throughout this volume.

II. CHEMICAL STRUCTURE

To an organic chemist an amino acid is any of a large group of compounds which contains one or more amino groups and one or more acid groups. To the biochemist, however, this term applies only to a very limited number of compounds, all of which have in common an amino group in the α -position to a carboxyl group, i.e.,

$$\begin{array}{c} \text{NH}_2 \\ | \\ \text{R}-\text{C}-\text{COOH} \\ | \\ \text{H} \end{array}$$

the general structure R-C-COOH. In addition, they are all

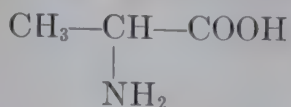
products of hydrolysis of proteins or are very closely related to these products.

In 1931 Vickery and Schmidt (238) set up certain standards to be met before an amino acid be recognized as a constituent of a protein hydrolysate. These included isolation by some worker other than the discoverer, characterization by suitable derivatives, establishment of structure by synthesis, and if possible, demonstration of utilization by the animal body.

Later Vickery (233) prepared an excellent resume of the status of the various amino acid components of proteins and separated them into four groups as follows: 1) Eighteen amino acids concerning which there is no doubt whatever; 2) Seven amino acids which occupy a special position because of their narrow range of distribution or for other reasons; 3) Five amino acids found in plants that may be constituents of proteins; and 4) Seventeen amino acids for which claims have not been substantiated.

It is beyond the scope of this chapter to consider all of these compounds. It is intended here to discuss only those substances which are of general practical importance to the protein chemist and nutritionist, although some of them have not been established as constituents of protein hydrolysates. They are twenty-eight in number and are listed alphabetically.

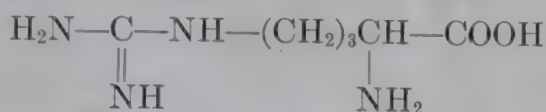
1. *Alanine* (α -aminopropionic acid)



In 1850, Strecker (219) in an attempt to synthesize lactic acid treated acetaldehyde ammonia with a mixture of hydrocyanic and hydrochloric acids and obtained instead a crystalline substance which he named alanine. The discovery that this substance is a constituent of most proteins did not come until some years later. In 1875, Schützenberger and Bourgeois (205) isolated from a silk hydrolysate a crystalline substance whose elementary analysis agreed with that of alanine. They characterized it no further and therefore it is Weyl (246) who is usually credited with the demonstration of the presence of alanine in protein hydrolysate. In 1888, he crystallized it from an acid hydrolysate of silk. He reported that it was optically inactive and that its decomposition point was identical with that of synthetic DL-alanine. He also prepared and analyzed the copper salt (247). Fischer and Skita (101) repeated the isolation and obtained an optically active alanine, the structure

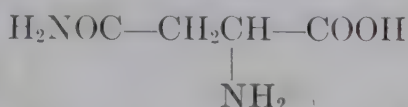
of which they established irrevocably by conversion to lactic acid.

2. *Arginine* (α -amino- δ -guanidovaleric acid)



Schulze and Steiger (212) first isolated arginine in 1886 from etiolated lupine seedlings. It was not until 1895, however, that Hedin (125) obtained it from an acid hydrolysate of a protein. Two years later Schulze and Winterstein (213) established its structure by demonstrating that the products of alkaline treatment were ornithine and urea. This structure was confirmed by Sørensen (217) in 1910, who synthesized the molecule from benzoyl ornithine by condensation with cyanamide and subsequent removal of the benzoyl group by acid hydrolysis. Gulewitsch (120) in 1899 was the first to prepare free crystalline arginine.

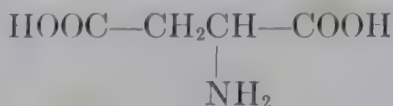
3. *Asparagine* (α -aminosuccinamic acid)



This substance was isolated by Vauquelin and Robiquet (232) from the juice of asparagus in 1806. Although it was long considered to be a constituent of proteins it could not be isolated from acid or basic hydrolysates because of the ease with which it is converted to aspartic acid. Actual proof of its presence in the protein molecule came in 1932 when Damodoran (69) isolated it from an enzymatic hydrolysate of edestin. Its structural relationship to aspartic acid was shown by Piria (184) in 1848 and this structure was confirmed by Piutti (186) who prepared it from the β -monomethyl ester of L-aspartic acid by the action of alcoholic ammonia.

Asparagine, with glutamine, is found in high concentration in etiolated seeds. Asparagine is converted to aspartic acid and ammonia by the enzyme asparaginase.

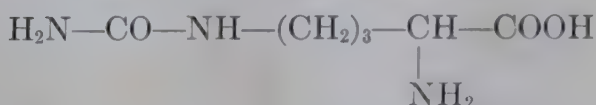
4. *Aspartic acid* (α -aminosuccinic acid)



Although aspartic acid had long since been obtained from asparagine by acid hydrolysis it was first shown to be a constituent

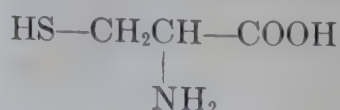
of protein hydrolysates by Ritthausen (194) in 1868. Its structure was established by Piria (184) in 1848. It was first synthesized by Dessaignes (72) who obtained it by the dry distillation of the ammonium salts of maleic, fumaric, and malic acids. Confirmation of structure by synthesis did not come however until 1887 when Piutti (187) prepared the inactive compound by reduction of oximinosuccinic acid diethyl ester. The synthetic compound was shown to be identical with a preparation obtained from optically active aspartic acid by racemization with hydrochloric acid at 180°.

5. *Citrulline* (δ -carbamidonorvaline)



This amino acid was isolated by Koga and Odake (145) from the juice of the watermelon in 1914 and later from a casein hydrolysate by Wada (243). Its importance to the amino acid chemist lies, however, not in its being a constituent of proteins which is not well established but in the fact that it is almost certainly an intermediate in the formation of urea in the animal body (149). Wada (242) not only determined the structure of citrulline by converting it to ornithine by alkaline hydrolysis but also confirmed this structure by synthesis from α -monobenzoylornithine and urethane. Citrulline may be conveniently prepared from arginine by boiling with an equimolecular quantity of alkali in 2.8 *N* solution (108). The amino acid so obtained is optically inactive as was the material isolated by Wada.

6. *Cysteine* (β -mercaptoalanine)

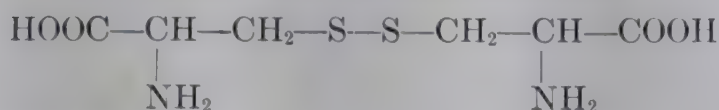


Cysteine was obtained from cystine by reduction with tin in hydrochloric acid by Baumann (19) in 1883. The researches of Neuberg (173) and Friedmann (112) contributed greatly to the elucidation of its structure and in 1904 the suggested structure was proved by Erlenmeyer (86) who synthesized it from benzoylserine. Cysteine in neutral or basic solution is readily oxidized to cystine by the oxygen of the air.

Cysteine has not definitely been shown to be a constituent of proteins although its presence is indicated by the fact that a few

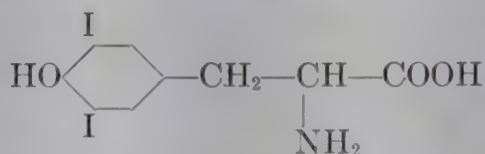
native proteins give a positive nitroprusside test. Mirsky and Anson (169) have presented evidence to show that the sulphhydryl content of proteins does not come from reduction of the disulfide, but exists *per se*. Cysteine is a constituent of the physiologically active tripeptide, glutathione, and in this or other combinations plays a role in oxidation-reduction reactions of the body.

★7. *Cystine* (β,β' -dithiobisalanine)



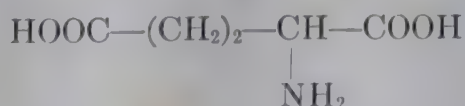
Cystine was the first amino acid to be discovered, having been isolated by Wollaston in 1810 from a urinary calculus. It was not until 1899, however, that Mörner (170) reported its isolation from an acid hydrolysate of horn. Its relationship to cysteine was established in 1883 (19) and its structure was verified by Erlenmeyer (85) who obtained it from benzoylserine without the isolation of the intermediate cysteine. The cystine content of the keratins is very high and, by cross linking, this amino acid is thought to be responsible for the hardness and toughness of these proteins.

8. *Diiodotyrosine* [β -(3,5-iodo-4-hydroxyphenyl) alanine]



In 1896 Drechsel (75) isolated diiodotyrosine from a species of coral, *Gorgonia Calvolinci*, and called it iodogorgoic acid. Since the determination of structure and synthesis of this compound by Wheeler and Jamieson (248) in 1905 the two terms have been used interchangeably. Diiodotyrosine is easily converted to tyrosine by warming with hydriodic acid. Apart from its presence in certain marine animals it occurs only in the thyroid gland protein and is of importance as a possible precursor of thyroxine.

9. *Glutamic acid* (α -aminoglutaric acid)

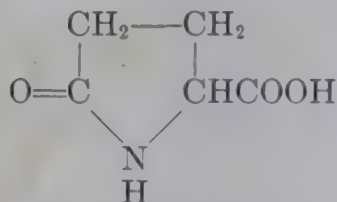


Ritthausen (193) was the first to isolate glutamic acid having obtained it in 1866 from an acid hydrolysate of wheat gluten.

Later he obtained it from other proteins, prepared its barium, copper, and silver salts, and established its empirical formula (194). Dittmar (73) reduced the product obtained by the action of nitrous acid on glutamic acid and Markownikoff (166) proved that this reduction product was identical with glutaric acid. Wolff (256) in 1890 synthesized glutamic acid from levulinic acid, deaminated it with nitrous acid and proved the hydroxy acid he obtained to be identical with that derived from the natural product.

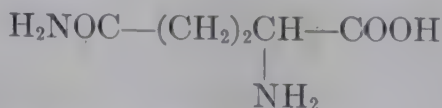
Glutamic acid occurs widely in nature and in especially high concentration in cereal proteins. It is of considerable economic importance because its monosodium salt is used widely as a condiment.

In solution over a wide pH range glutamic acid is converted by boiling to optically active pyrrolidonecarboxylic acid (pyroglutamic acid) (253). At pH 4 equilibrium is reached when about 98% of the glutamic acid has been lactamized.



Solid L-glutamic acid upon heating to 160°, melts and is converted to L-pyrrolidonecarboxylic acid; while increasing the temperature to 190° racemizes the product (7, 14).

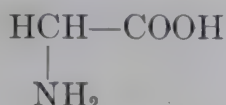
10. Glutamine (α -aminoglutaramic acid)



Glutamine (13), like asparagine, was isolated and characterized long before its presence in the protein molecule was established. It was obtained from beet juice in 1883 (209) and while it was long agreed that most of the glutamic acid found in proteins existed as glutamine positive proof was not forthcoming until 1932 when Damodoran, Jaaback and Chibnall (70) isolated it from an enzymic digest of gliadin. The structure of glutamine was clarified in 1900 by Schiff (202) and it was converted to glutamic acid by Micko (168) in 1908 by hydrolysis with barium hydroxide. Its synthesis from L-glutamic acid was accomplished by Bergmann and his collaborators (30) by application of their ingenious carbobenzoxy synthesis.

Glutamine upon heating in solution to 100° at pH 6.5 is rapidly converted to the ammonium salt of L-pyrrolidonecarboxylic acid (235). Upon treatment with nitrous acid in acetic acid it yields nitrogen equivalent to 180% of its amino nitrogen (13), a unique property in that amides including asparagine react with nitrous acid only in the presence of strong mineral acids.

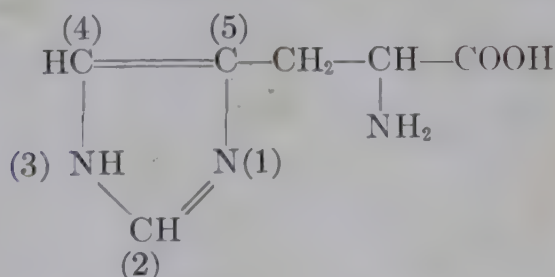
11. *Glycine* (aminoacetic acid)



Glycine, the simplest of protein constituents, is the only amino acid which contains no asymmetric carbon atom. It was isolated by Braconnot (37) in 1820 from a sulfuric acid hydrolysate of gelatin. It was probably the first amino acid to be obtained from a protein hydrolysate in a pure state. Cahours (44) in 1857 established the structure of the molecule and a year later he synthesized it from chloroacetic acid and ammonia.

Glycine, apart from its role as a constituent of most proteins, is of great interest as one of the precursors of creatine (33) which plays an important part in muscle contraction.

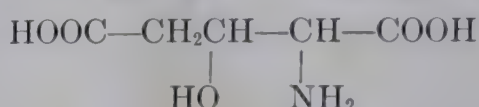
12. *Histidine* (α -amino- β -5-imidazolepropionic acid)



This amino acid was discovered in 1896 by two investigators independently. Kossel (148) obtained it from protamines while Hedin (126) isolated it from the acid hydrolysates of other proteins. Pauly (181) established that histidine contained an imidazole (glyoxaline) group and that the molecule through this group coupled with diazotized sulfanilic acid to yield a highly chromogenic compound. Its constitution was determined mainly by the work of Frankel (111), Pauly (181), and Knoop and Windaus (144). In 1911 Pyman (191) synthesized it from citric acid and resolved the racemic compound into its optically active isomers. Histidine, serine and threonine alone of the free amino acids give the biuret test.

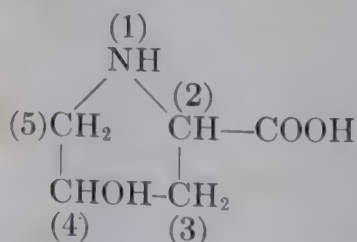
Histidine is a constituent of most proteins. Apart from this it is of importance as the precursor of histamine which is a powerful vasodilator and stimulator of gastric secretion. It is also a constituent of carnosine (β -alanyl-L-histidine) (20) a compound which is found in muscle and which also has a marked depressor action on blood pressure.

13. *Hydroxyglutamic acid* (α -amino- β -hydroxyglutaric acid)



This amino acid is of interest largely because of the controversy concerning its existence. Dakin (63) first reported its isolation in 1918 and gave evidence that the hydroxyl group was attached in the β -position. It was generally accepted as a protein constituent until Nicolet and Shinn (178), by their periodic acid oxidation method for the estimation of β -hydroxyaminoacids, were unable to demonstrate the presence of any β -hydroxyglutamic acid in hydrolysates of casein, lactalbumin or β -lactoglobulin. Dakin (66) has also stated that it seems certain that the acid he isolated cannot be a β -hydroxy acid since he was unable to convert it to ketogulonic or glutaconic acid derivatives and suggests that the hydroxyl group is attached to the γ -carbon. In the meantime Chibnall and co-workers (15) have reported that the material obtained by Dakin's isolation procedure is a mixture of aspartic acid, glutamic acid, dibasic dismutation products of cystine, serine and serine decomposition products. Clearly, hydroxyglutamic acid is not a constituent of proteins.

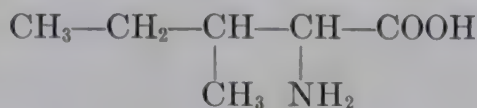
14. *Hydroxyproline* (4-hydroxy-2-pyrrolidinecarboxylic acid)



Fischer (91) in 1902 isolated hydroxyproline from an acid hydrolysate of gelatin. By means of phosphorus and hydriodic acid he reduced the hydroxyl group under conditions which yielded racemic proline. It remained then to establish the position of the hydroxyl group and although Leuchs (156) synthesized the compound in 1905 it was not until 1913 that he with Brewster (158)

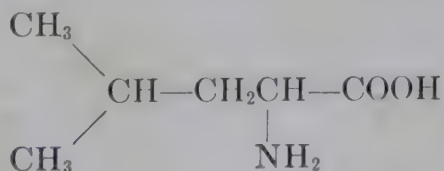
separated the proper isomer from the reaction mixture and showed it to be identical with the substance isolated from proteins. Later in 1919, Leuchs and Bormann (157) prepared the three remaining optical isomers. From the fact that no γ -lactone could be formed from acetyl-L-hydroxyproline Kaneko (140) deduced that the carboxyl and hydroxyl radicals are on opposite sides of the molecule.

15. *Isoleucine* (α -amino- β -methylvaleric acid)



Ehrlich (81) in 1904 isolated a substance from beet molasses and later from a blood fibrin hydrolysate which he found to have a chemical composition identical to that of leucine. Its properties, however, differed from those of leucine. In 1907 Ehrlich deduced the structure of isoleucine by degrading it to the corresponding amyl amine and amyl alcohol. At this time he reported the preparation of a mixture of L-isoleucine and the dextrorotatory diastereoisomer (d-alloisoleucine) from optically active valeraldehyde by a modified Strecker synthesis. He then separated the d-alloisoleucine in pure form by destroying the L-isoleucine by incubation with a pure culture of yeast. The same year Locquin (164) resolved synthetic inactive isoleucine into its optically active isomers, but it was not until 1931 that Abderhalden and Zeisset (11) prepared and characterized all four optically active forms of the molecule.

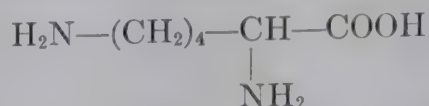
✓ 16. *Leucine* (α -aminoisocaproic acid)



Leucine was obtained as a hydrolytic product of proteins in an impure form by Proust (190) in 1819 and in 1820 by Braconnot (37) who gave it its name. Cahours (44) in 1858 showed that leucine must be an α -aminocaproic acid but did not establish the structure of the carbon chain. The first reported synthesis is that of Limpricht (161) in 1855 who applied the Strecker method to valeraldehyde. It is extremely doubtful, however, that Limpricht obtained anything approaching pure leucine since the aldehyde he used boiled at 97° , while the accepted boiling point for isovaleraldehyde is 92.5 and that for the straight chain isomer 103.4° . The constitu-

tion of leucine was finally established in 1891 by Shulze and Likiernik (210) who showed that the compound prepared by the Strecker synthesis from isovaleraldehyde was identical with DL-leucine obtained from the optically active compound by racemization.

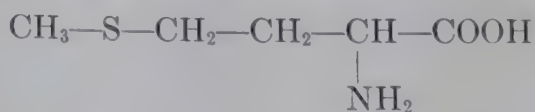
✓ 17. *Lysine* (α - ϵ -diaminocaproic acid)



Drechsel (74) in 1889, isolated from the phosphotungstic acid precipitate of a casein hydrolysate a substance which he called "lysatine" and which was later shown to be a mixture of lysine and arginine. Later Drechsel and his assistants reported the preparation of the dihydrochloride of a base which analyzed as diaminocaproic acid and appeared to be a homologue of ornithine. This was supported by the observation of Ellinger (82) that pentamethylenediamine was obtained from lysine by anaerobic putrefaction. The question of structure was finally settled in 1902 when Fischer and Weigert (103) synthesized it from γ -cyanopropylmalonic ester and demonstrated their product to be identical with the racemized protein constituent. It was not until 1928 that lysine was first obtained as the crystalline base by Vickery and Leavenworth (237).

No lysine can be obtained from proteins which have been deaminized with nitrous acid indicating that both amino groups of lysine are not involved in the peptide chain. For some time it was considered that all free amino groups of proteins are due to lysine, but more recent evidence indicates that in certain proteins other amino acids also contribute terminal amino groups (200). Lysine was one of the first amino acids to be shown to be necessary for growth of animals (180).

18. *Methionine* (α -amino- γ -methylmercaptobutyric acid)

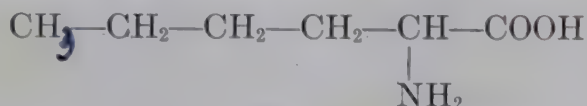


Mueller (171), in 1922, announced the discovery of a new sulfur containing amino acid which he obtained from the mercuric sulfate precipitate of an acid hydrolysate of casein. He established the empirical formula and by synthesis eliminated the possibility of the new compound being ethyleysteine. Barger and Coyne (16) found that the molecule contained one methiol group and reasoned

that it must be a methiol derivative of one of the butyric acids. Accordingly, in 1928, they synthesized what appeared to them to be the most likely isomer by application of the Strecker reaction to β -methiolpropionaldehyde. They prepared the thiohydantoin and the picrolonate of the synthetic product, and the melting points were found to be identical with the corresponding derivatives of the isolated amino acid. Windus and Marvel (254) in 1931 resolved DL-methionine through the brucine salts of the formyl derivative and supplied further proof of the identity of the synthetic with the natural material by comparison of suitable derivatives.

It is of interest that the presence of this amino acid was first detected because of its requirement for growth by hemolytic streptococcus. Its concentration was followed by its effect on the growth of this organism.

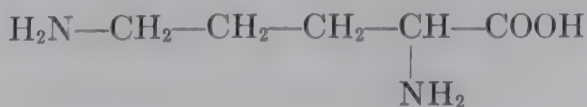
19. *Norleucine* (α -aminocaproic acid)



Norleucine probably does not occur in proteins, and its existence therein has been a subject of some controversy. Its isolation from nerve proteins was first reported by Thudichum (221) in 1901. Subsequently, Abderhalden and Weil (10) in 1912 isolated a similar substance from nerve tissue and considered it to be identical with L- α -aminocaproic acid. In 1933, Schmidt (203) recommended that it be added to the list of accepted amino acids. Recently, on the basis of chromatographic and bacteriological data and examination of derivatives it has been reported (56) that Thudichum's compound was DL-leucine. Racemization might be expected to have occurred since Thudichum employed alkaline hydrolysis in his preparative procedure. The first synthesis of norleucine was that of Hüfner (134) who prepared it from α -bromocaproic acid and ammonia in 1870.

Although there is a strong probability that norleucine is not a protein constituent, Burroughs, Burroughs, and Mitchell (42) report that it will replace leucine in maintaining nitrogen balance in the adult rat.

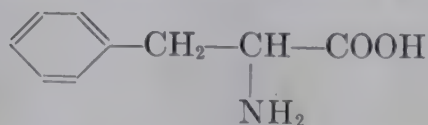
20. *Ornithine* (α - δ -diaminovaleric acid)



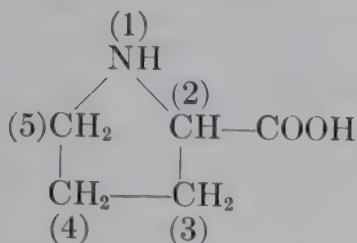
In 1877, Jaffé (136) fed chickens benzoic acid and isolated from their excretion products a substance which he called ornithuric acid and which he was able to convert by hydrolysis to salts of ornithine. Shulze and Steiger (211) in 1886, immediately after their isolation of arginine, observed that alkaline hydrolysis decomposed their new amino acid into carbon dioxide, ammonia and a strong base which they isolated as the salt of mineral acids. It was not until 1897 that Shulze and Winterstein were able to demonstrate that this substance was identical with Jaffé's ornithine. Jaffé surmised that ornithine must be a diaminovaleric acid and much later in 1900 Ellinger (82) established the position of the amino groups by obtaining tetramethylenediamine and carbon dioxide by the action of putrefactive bacteria. It remained, then, to establish the position of the carboxyl group and this Fischer (90) did in 1901 by synthesizing the compound from phthalimido-propylmalonic ester. The free base which is rather unstable was first prepared by Vickery and Cook (236) in 1931.

While ornithine is apparently not a constituent of proteins, it does occur in the antibiotic, tyrocidine. It is of great interest to the amino acid and protein chemist because of its position in the Krebs cycle (149) for urea production in the animal body.

21. *Phenylalanine* (α -amino- β -phenylpropionic acid)

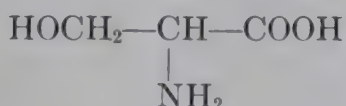


Schulze and Barbieri (206) in 1879 first isolated phenylalanine from the sprouts of lupine. Posen (189) the same year announced the synthesis of phenylalanine from bromophenylpropionic acid and ammonia but the physical data he gave to identify his product indicate that it was not phenylalanine. Schulze and Barbieri (208) four years after their initial discovery isolated the new amino acid from acid and alkaline hydrolysates of squash seed proteins and thus showed it to be a protein constituent. These same authors (207) had already established the correct empirical formula, demonstrated that oxidation yielded benzoic acid and that dry distillation gave what appeared to be phenylethylamine. They concluded that their amino acid was one of the phenylamino-propionic acids. Erlenmeyer and Lipp (87) in 1882 synthesized phenylalanine from phenylacetaldehyde by the Strecker method and Schulze and Barbieri (208) proved that their protein constituent was structurally identical with the synthetic compound.

22. *Proline* (2-pyrrolidinecarboxylic acid)


In an attempt to fix the position of the carboxyl group in hygric acid (1-methyl-2-pyrrolidinecarboxylic acid) Willstätter (252) in 1900 synthesized 2-pyrrolidinecarboxylic acid through bromopropylmalonic ester. A year later Fischer (96) announced its discovery as a protein constituent having isolated it from casein by application of his ester distillation method. In 1904 he gave it the name of proline. Although Fischer isolated a small quantity of the optically active amino acid, he found that the copper salt of the racemic compound was much more insoluble and that some of the proline had become racemized during his isolation procedure. Accordingly, he isolated this inactive compound and proved its identity with synthetic 2-pyrrolidinecarboxylic acid which he had prepared independently from phthalimidopropylmalonic ester (90).

Proline is the only alcohol soluble amino acid and most of the alcohol soluble proteins have a high proline content.

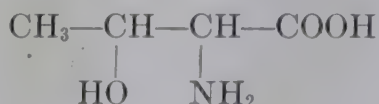
 23. *Serine* (α -amino- β -hydroxypropionic acid)


In 1865 Cramer (57) isolated from silk a protein which he called sericine. He hydrolyzed this new protein with sulfuric acid and from the hydrolysate, after the removal of tyrosine, obtained a new amino acid. He named this new substance serine, determined that it had many of the chemical properties of Strecker's alanine and established that its formula differed from that of alanine by one oxygen atom. He then established its structure as one of the amino hydroxypropionic acids by converting it to glyceric acid with nitrous acid. The relative positions of the amino and hydroxyl groups were not established until 1902 when Fischer and Leuchs (98) synthesized the amino acid by the action of ammonia and hydrocyanic acid upon glycolic aldehyde and proved it to be identical with optically inactive serine previously isolated from a protein hydrolysate by Fischer's ester distillation method (102). The nat-

ural serine had been racemized during the isolation procedure. Fischer and Leuchs also converted serine to alanine by reduction with red phosphorus and hydriodic acid.

Apart from its important role as a constituent of most proteins serine is also reported to be a component of brain cephalin (105). In certain proteins it is known to be in ester linkage with phosphoric acid (154, 162).

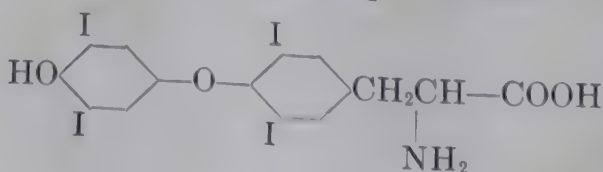
24. *Threonine* (α -amino- β -hydroxybutyric acid)



Threonine, the last of the generally accepted amino acids to be discovered, was isolated by Rose and his associates (195) in 1935 from an acid hydrolysate of blood fibrin. This group of investigators deduced that they were dealing with an aminohydroxybutyric acid. They confirmed this and also established the position of the amino group in the molecule by reducing the unknown compound to L- α -aminobutyric acid. The position of the hydroxyl group was established by elimination by comparison to the known γ -hydroxy acid and was later confirmed by oxidation of threonine to D(-)lactic acid (167). The aforementioned reduction and oxidation to known optically active compounds established the configuration of the groups around the α - and β -carbon atoms. Carter (45) in 1935 synthesized a mixture of the four isomers of α -amino- β -hydroxybutyric acid from crotonic acid and later West and Carter (244) separated from this mixture one of the isomers which they proved to be identical with the protein constituent.

The isolation of threonine was a direct result of Rose's attempts to satisfy the protein requirements of the growing rat with a mixture of crystalline amino acids. The fact that the two most recently discovered amino acids, threonine and methionine, were isolated as a result of their growth promoting effects on the living organism indicates the steadily increasing importance of amino acids in nutrition and of bioassay methods in biochemistry.

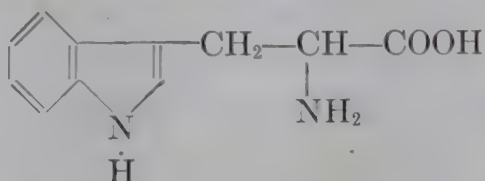
25. *Thyroxine* β -[3,5-diiodo-(3',5'-diiodo-4'-hydroxyphenoxy)phenyl] α -aminopropionic acid



Although it had long been known that there was present in the thyroid gland an active iodine-containing substance it was not until 1915 that Kendall (143) was able to isolate thyroxine from an alkali hydrolysate of the thyroid protein. Work on the structure of the new compound was begun by Kendall but it was Harrington (123) who finally established the structure and with Barger, in 1927, confirmed this structure by complete synthesis (124).

As far as is known thyroxine occurs naturally only in the thyroglobulin of the thyroid gland. It is formed in proteins that are iodinated in alkaline solution. Since it is the active principle of the thyroid, which, in general, controls the oxidative processes of the body, its importance cannot be overestimated.

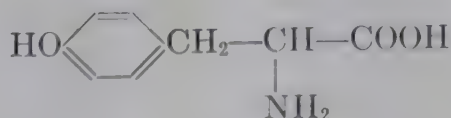
26. Tryptophan (α -amino- β -3-indolepropionic acid)



In 1901, Hopkins and Cole (132) isolated tryptophan from an enzymatic digest of casein. They established the correct empirical formula and showed that putrefactive action of bacteria on tryptophan produced indole, skatole and other derivatives containing the indole nucleus. Ellinger (83) synthesized 3-indoleacetic and 3-indolepropionic acids and found them to be identical with two of the previously isolated putrefactive products of protein (172, 199). He then concluded that tryptophan was most likely α -amino-3-indolepropionic acid and with Flamand (84) verified this in 1907 by synthesis from 3-indolealdehyde and hippuric acid.

Tryptophan occurs in many proteins but is a major constituent of none. It is interesting that the antibiotic peptide *gramicidin* contains 40% of tryptophan

27. Tyrosine [α -amino- β -(*p*-hydroxyphenyl) alanine]

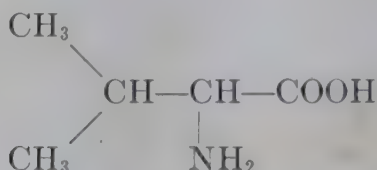


Liebig (160) in 1846 fused casein with alkali and from the fusion mixture isolated tyrosine. De la Rue (71) two years later obtained it from the cochineal insect and determined its empirical formula. Barth (17) showed that on alkaline fusion, tyrosine yields *p*-

hydroxybenzoic acid and acetic acid and was the first to suggest the correct structure. Evidence in favor of Barth's postulated structure was supplied by Baumann (18) in 1879 when he showed that tyrosine yields *p*-hydroxyphenylpropionic acid on bacterial decomposition. Erlenmeyer and Lipp (88) in 1883 synthesized tyrosine from *p*-aminophenylalanine and indicated its structural identity with the naturally occurring compound.

Tyrosine occurs widely in nature and is of great interest as the precursor of such physiologically important substances as adrenaline, thyroxine, and tyramine. Tyrosine, subjected to the action of air in the presence of the enzyme tyrosinase, is oxidized to a dark, weakly acidic pigment called melanin.

28. *Valine* (α -aminoisovaleric acid)



Valine was first isolated from aqueous extracts of liver, spleen, thymus and pancreas in 1856 by von Gorup-Besanez (118) who indicated it to be a member of the amino acid series. It was not until 1879, however, that Schützenberger (204) showed that it was a constituent of proteins by isolating it from an albumin hydrolysate. Without definite structural proof he indicated the new substance to be aminovaleric acid. Valine was first synthesized by Clark and Fittig (54) in 1866 by the action of ammonia on α -bromoisovaleric acid. The problem of structure, however, was not settled until Fischer (93) in 1906 resolved the synthetic product and showed that one of the optically active isomers so obtained was identical with the isolated compound.

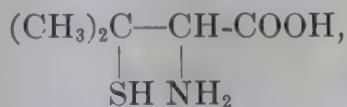
For more complete information concerning the history of the discovery of the amino acids the reader is referred to an excellent review prepared by Vickery and Schmidt (238).

Although, as was stated early in this section, many other amino acids have been reported as constituents of protein hydrolysates, only one of these appears to be of especial importance. In 1921 Van Slyke and Heller (229) reported that a phosphotungstic acid precipitate of a gelatin hydrolysate contained a basic substance in addition to the hexone bases ordinarily found. Later Van Slyke and his associates (230, 231) prepared the picrate and the hydro-

chloride of this unidentified substance, established its composition as being that of hydroxylysine and showed that upon periodate treatment it consumed one atom of oxygen with formation of one mole each of ammonia and formaldehyde. Thus it was established that the hydroxy and amino groups are adjacent and that one of them must be terminal. Since there was no lactone formation, these investigators considered it likely that neither group occupied the γ position to the carboxyl and therefore there could be no branching of the chain. From these observations, it was concluded that the unknown amino acid must have one of the two following structures, $\text{CH}_2(\text{NH}_2) \cdot \text{CH}(\text{OH}) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$,* or, $\text{CH}_2(\text{OH}) \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$. This contention has yet to be established by synthesis and resolution.

Of steadily increasing importance to the biochemist are the naturally occurring D-amino acids which have been found to be constituents of antibiotics and other microbiological metabolic products. However, none of these substances have been established as protein constituents. Kögl (147) reported that certain amino acids, especially glutamic acid, exist partially racemized in the protein of tumorous tissues. Subsequent researches in several laboratories (249, 2, 50) have, however, failed to verify his findings.

The amino acid moiety of penicillin, penicillamine



most of the phenylalanine isolated from tyrocidine (116) and part of the valine found in gramicidin (51) possesses the D configuration on the α -carbon atom. In addition, Ivanovics and Bruckner (135) have found that one component of the capsule of the anthrax bacillus and of many strains of the mesentericus group of bacilli is a polypeptide composed largely of D-glutamic acid, while Bovarnick (35) has demonstrated the formation of an extracellular polypeptide consisting entirely of D-glutamic acid. Wieland (251) has isolated 4-allohydroxyproline from phalloidine, the highly toxic peptide obtained from the fungus, *Amanita phalloides*.

The following table of properties includes some of the salts of the amino acids since it is in the form of these salts that the amino acids are most readily available (Table I).

* Hydroxylysine has been shown to have the δ -hydroxy structure (Sheehan, J. C., and Bolhofer, W. A.: *J. Am. Chem. Soc.*, 72: 2469, 2472 (1950)).

TABLE I

Amino acid	Empirical Formula	M.W.	C	H	N	
Alanine	$C_3H_7O_2N$	89.07	40.42	7.93	15.73	
Arginine	$C_6H_{14}O_2N_4$	174.14	41.35	8.10	32.18	
Arginine HCl	$C_6H_{15}O_2N_4Cl$	210.64	34.18	7.18	26.59	Cl-16.80
Asparagine	$C_4H_8O_3N_2$	132.06	36.35	6.11	21.20	
Aspartic Acid	$C_4H_7O_4N$	133.06	36.07	5.30	10.53	
Citrulline	$C_6H_{13}O_3N_3$	175.14	41.09	7.48	23.92	
Cysteine	$C_3H_7O_2NS$	121.12	29.69	5.83	11.57	S-26.47
Cysteine HCl	$C_3H_8O_2NSCl$	157.59	22.85	5.12	8.88	S-20.34 Cl-22.50
Cystine	$C_6H_{12}O_4N_2S_2$	240.23	29.97	5.03	11.66	S-23.69
Diiodotyrosine	$C_9H_9O_3NI_2$	432.91	24.97	2.10	3.24	I-58.63
Glutamic Acid	$C_5H_9O_4N$	147.08	40.80	6.17	9.52	
Glutamine	$C_5H_{10}O_3N_2$	146.15	41.09	6.90	19.17	
Glycine	$C_2H_5O_2N$	75.05	31.98	6.71	18.67	
Histidine	$C_6H_9O_3N_3$	155.09	46.42	5.85	27.10	
Histidine HCl · H ₂ O	$C_6H_{12}O_3N_3Cl$	209.55	34.4	5.73	20.04	Cl-17.0
Hydroxylysine	$C_6H_{14}O_3N_2$	162.13	44.3	8.7	17.3	
Hydroxyglutamic Acid	$C_5H_9O_5N$	163.08	36.79	5.56	8.59	
Hydroxyproline	$C_5H_9O_3N$	131.08	45.77	6.93	10.68	
Isoleucine	$C_6H_{13}O_2N$	131.11	54.92	10.00	10.68	
Leucine	$C_6H_{13}O_2N$	131.11	54.92	10.00	10.68	
Lysine	$C_6H_{14}O_2N_2$	146.13	49.27	9.66	19.17	
Lysine HCl	$C_6H_{15}O_2N_2Cl$	182.64	39.40	8.28	15.32	Cl-19.42
Methionine	$C_5H_{11}O_2NS$	149.15	40.23	7.42	9.39	S-21.50
Norleucine	$C_6H_{13}O_2N$	131.11	54.92	10.00	10.68	
Ornithine	$C_5H_{12}O_2N_2$	132.16	45.44	9.15	21.20	
Ornithine HCl	$C_5H_{13}O_2N_2Cl$	168.64	35.58	7.77	16.60	Cl-21.20
Phenylalanine	$C_9H_{11}O_2N$	165.09	65.41	6.72	8.48	
Proline	$C_5H_9O_2N$	115.08	52.13	7.88	12.16	
Serine	$C_3H_7O_3N$	105.06	34.27	6.72	12.33	
Threonine	$C_4H_9O_3N$	119.07	40.30	7.62	11.76	
Thyroxine	$C_{15}H_{11}O_4NI_4$	776.77	23.17	1.43	1.80	I-65.35
Tryptophan	$C_{11}H_{12}O_2N_2$	204.11	64.67	5.93	13.72	
Tyrosine	$C_9H_{11}O_3N$	181.09	59.44	6.13	7.73	
Valine	$C_5H_{11}O_2N$	117.09	51.20	9.46	11.96	

III. CLASSIFICATION

A practical scheme for classification of the amino acids is according to their basicity. If a protein hydrolysate is placed in the center compartment of a three compartment cell and subjected to electro-dialysis at pH 5.5 according to the method described by Foster and Schmidt (107), the dicarboxylic amino acids, glutamic and aspartic acids will migrate to the anode, the diamino acids, arginine, lysine and histidine will move to the cathode compartment and the remaining amino acids being neutral with one carboxy and one amino group will remain in the center compartment. While histidine has two basic groups and will form a dihydrochloride its isoelectric point is 7.4 so that if the electro-dialysis is carried out at this pH it too will remain in the center compartment with the neutral amino acids.

Some indication of the basicity of the amino acids may be gained from a consideration of the stability of their ammonium salts. Glutamic and aspartic acids being strongly acidic form stable well characterized salts with ammonia. The monoaminomonocarboxylic acids form ammonium salts as shown by the fact that in most cases their solubility is increased by the presence of ammonia. However, the combination is extremely weak and the ammonia may be removed from an aqueous solution of these amino acids by concentration under reduced pressure. The strength of the two basic groups of histidine is shown by the fact that pyridine will convert the di- to the monohydrochloride whereas ammonia will convert either salt to the free base. Arginine and lysine are such strong bases, however, that while ammonia or pyridine will convert the di- to monohydrochloride, ammonia may be distilled from a solution of the monohydrochlorides.

Other methods of classification can, of course, be used. Five of the amino acids (phenylalanine, tyrosine, diiodotyrosine, thyroxine) and tryptophan) are aromatic in nature; four (proline, hydroxyproline, tryptophan, and histidine) are heterocyclic; while the remainder are aliphatic. Methionine and cystine contain sulfur, while diiodotyrosine and thyroxine contain iodine. There are six hydroxy acids (serine, threonine, hydroxylysine, hydroxyproline, tyrosine, and thyroxine), although the hydroxyl groups of tyrosine and thyroxine differ from those of the other members of the group in that they are phenolic in nature.

IV. PHYSICAL PROPERTIES

1. Optical Rotation

With the exception of glycine, all of the amino acids as they occur in nature are optically active. Those isomers which are constituents of proteins have in the past been referred to as the naturally-occurring or natural amino acids. Since the discovery of enantiomorphs of some of these components in certain antibiotics and other natural products, such designation is no longer tenable, and it becomes increasingly desirable to refer to the products of the hydrolysis of proteins and to their enantiomorphs as L- and D-amino acids respectively. The D-amino acids are not readily available except by resolution of the racemic or DL mixtures which have been obtained by synthesis or by racemization of the L-forms. In certain cases it is simpler to obtain the L-form of an amino acid by resolu-

tion of the synthetic mixture than to isolate it from a protein hydrolysate.

✓ There are three practical methods for the resolution of racemic amino acids:

a. Chemical resolution

An amino acid or its derivative is combined in a reversible manner with an optically active compound and the diastereoisomers so formed are separated by difference in solubility. The separated isomers are purified and by suitable chemical means are converted to the optically active amino acids. This method has the advantage that it is often possible to obtain both isomers by the same procedure. It suffers in that several steps are involved and the yields are often low.

b. Biological resolution

This is almost invariably a method for the preparation of the members of the D-series. Microorganisms sometimes show a high degree of specificity for the amino acids so that often only the L-form is metabolized. In this method the racemic amino acid is inoculated with a culture of a suitable microorganism and after incubation during which the L-form is metabolized the D-form is isolated from the culture medium.

c. Enzymatic method*

In this method advantage is taken of the fact that an enzyme system is highly specific. For example carbobenzoxy-DL-glutamic acid has been treated with aniline in the presence of activated papain (114). Carbobenzoxy-L-glutamic acid anilide was formed which was separated from unchanged carbobenzoxy D-glutamic acid and the latter was converted to optically pure D-glutamic acid. In this type of resolution both isomers may be obtained.

A second enzymatic method involves the use of D-amino acid

* Greenstein and co-workers (Fodor, P. J., Price, V. E., and Greenstein, J. P.: *J. Biol. Chem.*, 178: 503 (1949); Price, V. E., Gilbert, J. B., and Greenstein, J. P.: *Ibid.*, 179: 169 (1949); Gilbert, J. B., Price, V. E. and Greenstein, J. P., *Ibid.*, 180: 473 (1949); Greenstein, J. P., Gilbert, J. B. and Fodor, P. J.: *Ibid.*, 182: 451 (1950)) have developed an ingenious method of resolving N-acetylated racemic amino acids by means of the asymmetric enzymatic hydrolysis of the L-acylated isomer. The peptidases catalyzing this reaction occur in rat and hog kidney. The acetyl and chloroacetyl derivatives of the amino acids are employed. Amino acids that have been resolved in this manner include alanine, methionine, valine, threonine, isoleucine, serine, leucine, and aspartic and glutamic acids. Phenylalanine, tyrosine and tryptophan have been resolved into their optical isomers by subjecting their N-chloroacetylated derivatives to asymmetric hydrolysis by purified beef carboxypeptidase.

oxidase to convert the D-component of a racemic mixture to the corresponding keto acid. The unchanged L-isomer may then be separated optically pure (23, 76).

Isoleucine, hydroxyproline, and threonine each possess two asymmetric carbon atoms and therefore each exists in four optically active forms.⁷ The diastereoisomers of the protein constituent are generally designated by the prefix allo-. In each case the four isomers have been prepared (11, 157, 244, 245) and their optical rotation determined.

For reproducibility, the optical rotation of an amino acid must be taken under carefully controlled conditions. The rotation varies with concentration and temperature and particularly with hydrogen ion concentration. As a result several of the amino acids (tryptophan, histidine, leucine, serine, aspartic acid, methionine) change the direction of their rotation with change in pH. The rotation is sensitive to small amounts of acid or base in the region of neutrality but is almost unaffected by relatively large changes when several moles of acid or base are present. For this reason, and because of the insolubility of some of the amino acids at neutrality, it is often desirable to make this determination in the presence of an excess of hydrochloric acid.

While biochemists in the past have leaned heavily on specific rotation as an indication of purity of amino acids it is not an especially reliable criterion for two reasons.⁸ First, with a few notable exceptions the specific rotations of the amino acids are quite low and therefore subject to large error in measurement and second, an impurity or mixture of impurities with a rotation close to that of the amino acid in question will remain undetected.

Lutz and Jirgensons (165) measured the rotations of many of the amino acids in acid and alkaline solutions. The data so obtained were used to indicate whether an amino acid was a member of the D- or the L-series.⁹ When the measurements are taken on the acid plateau, those amino acids whose rotations become more positive with increasing acid concentration are members of the L-series and conversely those whose rotations become more negative with increasing hydrogen ion concentration belong to the D-series.¹⁰

The specific rotations, $[\alpha]_D$ of the L-amino acids are given in Table II.

$[\alpha]_\lambda^t = 100\alpha/lc$ in which $[\alpha]_\lambda^t$ is the specific rotation at the wave length, λ , and temperature, t , α is the observed rotation, l is the length of the polarimetric tube in decimeters, and c is the concentration of the substance in gm. per 100 milliliters of solution.

TABLE II
SPECIFIC ROTATION OF THE L-AMINO ACIDS

	Temp., Degrees	Concen- tration gm/100 ml of Solution	Solvent	Moles of Acid or Base per Mole Amino Acid	$[\alpha]_D$	Ref. No.
Alanine	25	2.06	6 NHCl	26	+13.70	80
	25	10.03	5.97 NHCl	5.3	+14.48	80
	25	10.00	Water	0	+ 2.42	80
Arginine	20	1.78	3 NNaOH	15	+ 3.0	165
	23.3	1.65	6 NHCl	63	+27.58	*
	20	3.48	Water	0	+12.5	165
Aspartic Acid	20	0.87	0.5 NNaOH	10	+11.8	165
	24	2.02	6 NHCl	39	+24.6	77
	18	1.33	Water	0	+ 4.7	165
Asparagine	18	1.33	3 NNaOH	30	- 1.7	165
	20	2.24	3.4 NHCl	20	+34.26	21
	20	1.41	Water	0	- 5.30	21
Citrulline	20	11.23	2.5 NNaOH	3	- 6.35	21
	21-23	5.00	0.3 NHCl	1	+17.9	117
	21-23	5.00	Water	0	+ 3.5	117
Cysteine	26	12.1	NHCl	1	+ 7.6	222
Cystine	24	1.0	NHCl	25	-214.4	224
	18.5	0.4	0.2 NNaOH	12	-70.0	165
Diiodotyrosine	20	5.08	1.1 NHCl	9.5	+ 2.89	6
	20	4.41	13.4 NNaOH	132	+ 2.27	6
Glutamic Acid	25	7.01	1.73 NHCl	3.63	+31.71	*
	22.4	1.00	6 NHCl	87	+31.2	77
	18	1.47	Water	0	+11.5	165
Glutamine	18	1.47	NNaOH	10	+10.96	165
	22	3.6	Water	0	+ 5.0	70
	25	1.00-4.05	6.1 NHCl	25-100	+13.34	80
Histidine	25	0.75-3.77	Water	0	-38.95	80
	20	0.77	0.5 NNaOH	10	-10.9	165
Hydroxy- proline	20	1.31	NHCl	10	-47.3	165
	22.5	1.00	Water	0	-75.2	77
	20	0.65	0.5 NNaOH	10	-70.6	165
Hydroxypro- line (allo) (b)	18	2.62	Water	0	-58.1	80
Isoleucine	20	5.09	6.1 NHCl	15	+40.61	164
	20	3.10	Water	0	+11.29	164
	20	3.34	0.33 NNaOH	1.3	+11.09	81
Isoleucine (allo)	20	3.87	6 NHCl	20	+38.1	11
	20	2.00	Water	0	+14.0	11
Leucine	25	2.00	6 NHCl	39	+15.20	80
	25	9.075	4.5 NHCl	6.5	+13.91	*
	25	2.00	Water	0	-10.57	80
Lysine	20	1.31	3 NNaOH	30	+ 7.6	165
	23	2.00	6 NHCl	43	+25.9	77
	25	1.64	6.08 NHCl	5.42	+25.72	*
Methionine	20	6.50	Water	0	+14.6	77
	25	0.80	Water	0	- 8.11	80
Methionine (D)	20	5.00	3 NHCl	8.92	+23.40	76
	25	0.80	0.2 NHCl	4	-21.18	80
	25	0.80	Water	0	+ 8.12	80
Ornithine	25	0.80	0.6 NNaHCO ₃	11	- 7.47	80
Phenylalanine	20	0.84	0.45 NHCl	9	+14.1	165
Phenylalanine (D)	20	1.93	Water	0	-35.14	100
	20	3.81	5.4 NHCl	23	+ 7.07	99

* Unpublished data by Dunn, M. S., and coworkers.

TABLE II (continued)

	Temp., Degrees	Concen- tration gm/100 ml of Solution	Solvent	Moles of Acid or Base per Mole Amino Acid	$[\alpha]_D$	Ref. No.
Proline	20	0.57	0.5 NHCl	10	-52.6	165
	23.4	1.00	Water	0	-85.0	77
	20	2.42	0.6 N KOH	3	-93.0	104
Serine	25	9.34	NHCl	1	+14.95	97
	20	10.41	Water	0	-6.83	97
	26	1.09†	Water	0	-28.3	244
Threonine	26	1.63†	Water	0	-9.1	245
Threonine (allo)	—	3†	0.13 N NaOH in 70% ethanol	3	-4.4	106
Tryptophan	20	1.02	0.5 NHCl	10	+2.4	165
	22.7	1.00	Water	0	-31.5	77
	25	2.07	Water	0	-32.15	*
Tyrosine	20	2.42	0.5 N NaOH	4	+6.17	3
	25	4.005	6.08 NHCl	29.4	-1.165	*
	20	4.40	6.3 NHCl	28	-8.64	89
	18	0.90	3.0 N NaOH	60	-13.2	165
Valine	20	3.4	6 NHCl	20	+28.8	121
	20	3.58	Water	0	+6.42	93

† Grams of solute per 100 grams of solution.

2. Solubility

With the exception of proline which is soluble in alcohol (1.55% at 35°) (226), the free amino acids are insoluble in the common neutral solvents except water. Their solubility in water varies from 162 grams per 100 grams of water at 25° for proline to 0.011 grams per 100 grams of water at 25° for cystine. The common method of recrystallization is to dissolve the amino acid in water and precipitate it with one-half to five volumes of ethanol. Some of the amino acids are soluble in glacial acetic acid. A mixture of tyrosine and leucine may be separated by extracting the latter with this solvent (121).

Salt formation has a tremendous effect upon the solubilities of the amino acids in water and in polar organic solvents. In water the halides or alkali salts of all amino acids have increased solubility. The hydrochlorides and sodium salts of the monoamino monocarboxylic acids in general are extremely soluble in ethanol containing a small amount of water and some are quite soluble in absolute ethanol. The monohydrochlorides of the basic amino acids are insoluble in ethanol since the molecule still possesses an unneutralized basic group and exists in the form of the ethanol-insoluble zwitter ion. On the other hand when the remaining basic

group is neutralized the dihydrochloride acts as an organic acid and becomes alcohol soluble. The sodium salts of the basic amino acids are soluble in ethanol since they resemble strong organic amines. The monosodium salts of the dicarboxylic acids are insoluble in ethanol since they now resemble monoamino monocarboxylic acids, i.e., they possess one amino group and one unneutralized carboxyl group, while the disodium salts are more alcohol-soluble since these compounds resemble organic amines. The hydrochlorides, too, are soluble for they act as organic dicarboxylic acids.

Determination of the solubility of an amino acid has been used as the basis of a very valuable method of purity determination (see page 49). Schmidt and collaborators (67, 68, 225, 255), have determined the solubilities of many of the amino acids at various temperatures and have shown that thermodynamic equations may be used with relatively small error for calculating the solubility of an amino acid at a given temperature. Much of their solubility data are shown in Table III.

3. Melting Point

Most of the amino acids melt with decomposition. The temperature at which decomposition takes place varies a great deal with the rate of heating. Moreover the use of a sealed capillary will sometimes give a different result than is obtained in an open tube. Obviously the decomposition point of an amino acid is not a good criterion of high purity. It is however, often a means of detecting gross contamination or an aid in identification by mixed decomposition point with an authentic sample.

Establishing the true decomposition points and specific rotations of the amino acids under carefully controlled conditions has in the past been a very difficult undertaking. However, now that the reliable solubility phase method (see page 49) is applicable to most of the amino acids for determination of ultimate purity, it is possible to make these determinations on samples of known purity and in this way to establish their true values as well as the degree to which these values are affected by varying amounts of contaminants. Dunn, *et al.* (80) have used this method for establishing the purity of samples of L-leucine, L-histidine and D- and L-alanine whose specific rotations they studied.

Most decomposition takes place over a range of temperatures. The values listed below are the highest figures reported.

TABLE III
 SOLUBILITY AND DECOMPOSITION POINTS

	Solubility per 100 gm. H ₂ O at					Decomposition Point Degrees
	0°	25°	50°	75°	100°	
DL-Alanine (68)	12.11	16.72	23.09	31.89	44.04	295
L-Alanine (68)	12.73	16.51 (80)	21.79	28.51	37.30	297
DL-Arginine						238
L-Arginine						238
L-Arginine HCl						220 corr.
DL-Aspartic Acid (68)	0.262	0.775 (226)	2.000	4.456	8.594	280
L-Aspartic Acid (68)	0.209	0.500	1.199	2.875	6.893	270
L-Asparagine (24)	0.835	3.11 ^{28°}	7.90	24.58 ^{78°}	46.42	236
DL-Citrulline						219.5
L-Citrulline						222
L-Cysteine HCl						178
DL-Cystine		0.0326 ^{19°} (25)				260
L-Cystine (68)	0.005	0.011	0.024	0.052	0.114	261
DL-Diiodotyrosine (255)	0.015	0.034	0.077			200
L-Diiodotyrosine (68)	0.020	0.062	0.186	0.562	1.700	213
DL-Glutamic Acid	0.855	2.054	4.934	11.86	28.49	227
DL-Glutamic Acid H ₂ O		1.15 (226)				
L-Glutamic Acid (68)	0.341	0.843*	2.186	5.532	14.00	249
L-Glutamine		3.6 ^{18°}				185
Glycine (68)	14.18	24.99	39.10	54.39	67.17	292
DL-Histidine						286
L-Histidine		4.29 (80)				277
DL-Histidine HCl						272
L-Histidine HCl · H ₂ O						255
DL-Hydroxyproline						261
L-Hydroxyproline (225)	28.86	36.11	45.18	51.67 ^{65°}		270
DL-Hydroxyproline (allo)						250
L-Hydroxyproline (allo) (157)	25					241
DL-Isoleucine (68)	1.826	2.011 (226)	3.034	4.607	7.802	292
		2.229				
L-Isoleucine (68)	3.791	4.117	4.818	6.076	8.255	284
L-Isoleucine (allo) (81)		2.93 ^{20°}				281
DL-Leucine (68)	0.797	1.00 (226)	1.406	2.276	4.206	332
		0.991				
L-Leucine (68)	2.270	2.33 ^{35°} (226)	2.66 (108a)	3.823	5.638	337
		2.19 (80)				
L-Lysine						224
DL-Lysine HCl						264
L-Lysine 2HCl						201
DL-Methionine (68)	1.818	3.35 (226)	6.070	10.52	17.60	281
		3.381				
L-Methionine						283
DL-Norleucine (68)	0.843	1.15 (226)	1.727	2.861	5.229	327
		1.149				
DL-Ornithine						195
L-Ornithine						240
DL-Ornithine HCl						215
DL-Phenylalanine (68)	0.997	1.29 (226)	2.187	3.708	6.886	320
		1.411				
L-Phenylalanine (68)	1.983	2.965	4.431	6.624	9.900	284
DL-Proline						205
L-Proline (255)	127.4	162.3	206.7	239.0 ^{65°}		222
DL-Serine (68)	2.204	5.023	10.34	19.21	32.24	246
L-Serine						228
DL-Threonine		20.5 (226)		50.0 ^{80°} (244)		235
		20.1 (244)				
L-Threonine						253
DL-Threonine (allo)						252
L-Threonine (allo)						272
DL-Thyroxine						232
L-Thyroxine						237
DL-Tryptophan		0.25 ^{30°} (226)				285
L-Tryptophan (68)	0.823	1.14 (226)	1.706	2.795	4.987	282
		1.136				
DL-Tyrosine (255)	0.0147	0.351	0.0836			318
L-Tyrosine (68)	0.020	0.045	0.105	0.244	0.565	344
DL-Valine (68)	5.98	7.09	9.11	12.61	18.81	298
L-Valine (67)	8.34	8.85	9.62	10.24 ^{85°}		315

* Unpublished data from laboratory of Dunn, M. S.

4. Stability

The amino acids in general are exceptionally stable compounds. The author has observed that a solution of the ten growth essentials for the rat (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) may be autoclaved under an atmosphere of nitrogen at 120° for as long as 24 hours without detectable loss of any of the amino acids and without production of histamine or histamine-like substances. If, on the other hand, this same mixture of amino acids is heated in the dry form at 120° for three hours some charring occurs and a solution of the mixture has a powerful blood pressure depressing action.

With the exception of proline and tryptophan all of the amino acids appear to be quite stable under ordinary conditions of storage. Proline is hygroscopic and upon long storing sometimes becomes brown in color. Tryptophan upon aging often develops a yellow coloration.

Tryptophan¹ is readily destroyed by heating with a mineral acid while histidine, cystine and cysteine are unstable in alkali. Boiling alkali also effects the conversion of arginine to ornithine and brings about racemization of most of the optically active amino acids.

5. Taste

Kaneko (139) made an extended study of the taste of the amino acids as related to structure and concluded that amino acids having the general formula $RCH(NH_2)COOH$ are either tasteless or bitter when they belong to the L-series and sweet when they belong to the D-group. Notable exceptions to the rule are supplied by the optical isomers of alanine, both of which are sweet, as are the L-isomers of proline and hydroxyproline. Glycine and most of the racemic mixtures of the amino acids which are optically inactive are sweet tasting. Practically, the most important is the taste accentuating property of monosodium glutamate.

V. CHEMICAL PROPERTIES

The amino acids under suitable conditions may be made to undergo most of the reactions of both the organic amines and the organic acids. In addition, there are reactions which are peculiar to the α -amino acids alone. An excellent review of the chemical

¹ This is true in mixtures containing reducible substances. By itself, tryptophan is quite stable in acid.

properties of the amino acids is that by Clarke, in Gilman's Organic Chemistry.

1. Reversible Reactions

In this group will be considered those reactions from whose products the parent amino acids may be regenerated by simple hydrolysis.

a. Peptide bond formation

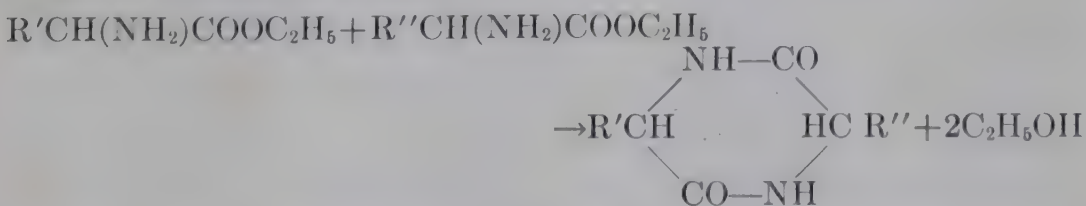
The most important reaction of the amino acids is, of course, condensation of the carboxyl group of one with the amino group of a second to form an amide type linkage known as the peptide bond. Protein synthesis, occurs in the living cell presumably under the influence of enzyme systems and the peptide bond is repeated to form long chains of amino acids residues with the elimination of a molecule of water for each amino acid added.



There is yet no method of effecting this reaction directly in vitro although peptides containing almost a score of amino acid residues have been synthesized by indirect methods which will be described in Chapter III. Recently polypeptides containing as high as 110 glycine residues have been obtained by subjecting glycine ethyl ester to various polymerizing conditions. The ethyl ester of alanine was also made to polymerize but its tendency to do so is much less marked (109).

b. Diketopiperazine formation

The peptide linkage is also involved in the formation of a second group of compounds, the diketopiperazine or piperazinediones, which are readily formed by refluxing an alcohol solution of the esters of the amino acids.



When R' and R'' are identical, the product of the reaction is often referred to as the anhydride of the parent amino acid. Abderhalden and Suzuki (9) prepared a series of esters of glycine and found that the reaction occurred most rapidly with the methyl

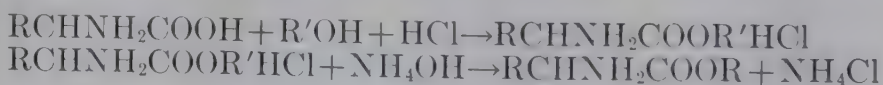
ester and most slowly with the benzyl ester. The yield of the reaction is doubled by the presence of a sugar which itself remains unchanged (151).

Recently it has been reported that the anhydride and mixed diketopiperazines are formed in excellent yield directly from the free amino acids by heating in glycol (201).

Small amounts of diketopiperazines have been isolated from protein hydrolysate (5) but the fact that the yield was greater the less completely the protein was hydrolyzed indicates that peptides and not amino acids were the precursors. This is substantiated by Lichenstein (159) who found that peptides were readily converted to diketopiperazines by heating with β -naphthol. Pierson, Giella and Tishler (183) have isolated the anhydride of methionine in low yield after autoclaving an aqueous solution of methionine hydantoin.

c. Esterification

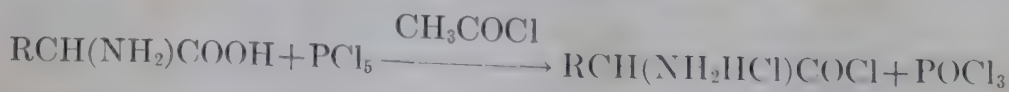
The amino acids may be esterified only in the presence of an equivalent of strong acid. The usual procedure is to suspend the amino acid in dry alcohol and with refrigeration pass in a stream of hydrogen chloride. The amino acid passes into solution as the hydrochloride is formed and often reprecipitates as the ester hydrochloride. The free ester may be obtained by neutralization followed by a suitable extraction procedure.



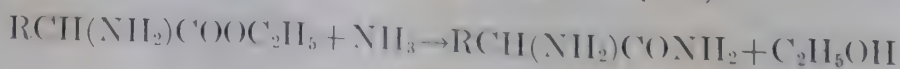
The free esters are moderately stable compounds which may be distilled but which hydrolyze readily in hot water.

d. Acyl halide and amide formations

The acyl halides of the free amino acids do not exist. They can be prepared only in the form of salts by shaking the amino acid with an acyl halide and phosphorus pentachloride (92).

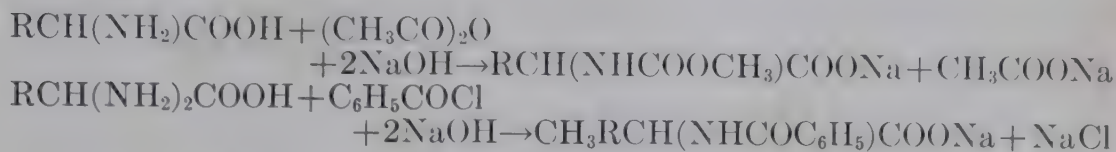


The amino acids do not readily react with ammonia to form the amides. These compounds can be prepared by treatment of the esters with alcoholic or anhydrous ammonia (146).



e. Acylation

Acylation of the amino acids takes place quite readily in the presence of alkali which not only suppresses the charge on the amino group but also neutralizes the mole of acid usually formed during the reaction.



The reaction mixture is neutralized with mineral acid and the product isolated by concentration or by extraction with a suitable organic solvent such as ethanol or ethyl acetate.

That alkali need not be present is shown by the fact that many of the amino acids may be acetylated with acetic anhydride in acetic acid (29) or formylated by formic acid alone (218). In the latter case the reaction proceeds much more smoothly in the presence of acetic anhydride (239) probably due to an anhydride exchange so that formic anhydride is the acylating agent. That the effect is not due merely to removal of water formed during the reaction is shown by the fact that the reaction proceeds rapidly in the presence of an excess of water.

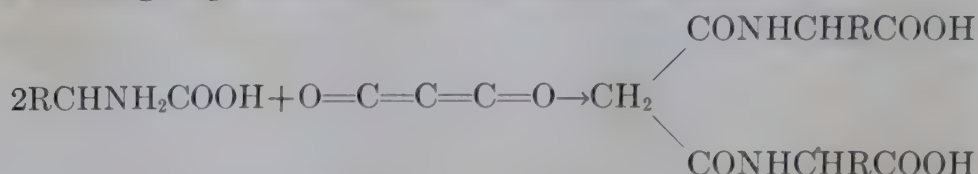
In a non-aqueous medium (29) an excess of acetic anhydride causes catalytic racemization of the amino acid. In aqueous solution very little racemization occurs with an excess of acetic anhydride (22) alone but it does take place rapidly if there is also present sodium hydroxide (240), sodium acetate (239), pyridine (239) or an azlactone (48). In all cases the racemization appears to take place through the formation of an intermediary azlactone.

Benzoylation with benzoyl chloride and sodium bicarbonate has been found to cause much more racemization than when sodium hydroxide is substituted for bicarbonate (49). It is believed that sodium hydroxide causes more rapid decomposition of excess benzoyl chloride which otherwise reacts with the benzoylated amino acid to form an easily racemized intermediate, probably an azlactone. As might be expected treatment of acetyl proline, a secondary amino acid incapable of azlactonization, with excess acetic anhydride and acetate causes no racemization.

Bergmann and Stern (28) developed a new acetylation method in 1930 when they found that the difference in reactivity of ketene and water and ketene and the amino group is so great that the amino acids may be acetylated in good yield. This is by far the best

method for the preparation of acetyl cysteine (185). Cahill and Burton (43) have found that racemization during acetylation with ketene occurs only when the reaction of the solution is on the acid side of neutrality.

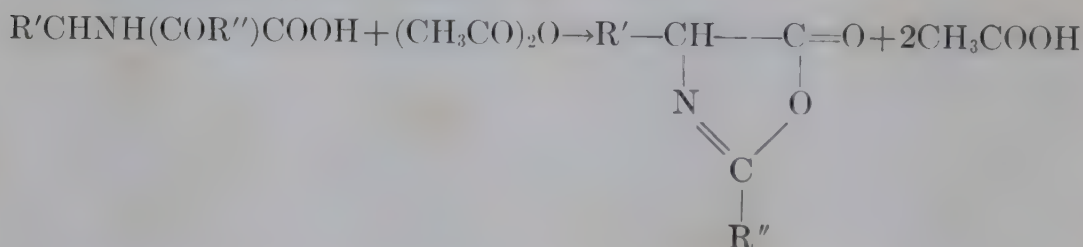
In general the amino acids react with carbon suboxide through their amino groups to form malonyl diamides (196).



In addition tyrosine may also react with its phenolic hydroxyl group to form the mono- or dimalonyl esters.

f. Azlactone formation

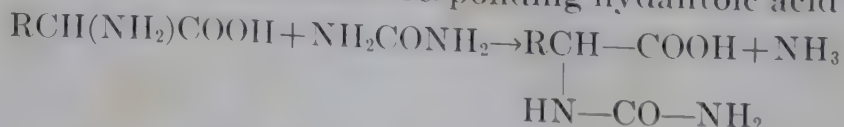
When acyl derivatives of amino acids are treated with molecular quantities of acetic anhydride (29) or benzoyl chloride (46) a molecule of water is removed and heterocyclic compounds, azlactones (oxazolines), are formed.



These products are never optically active unless R' contains an asymmetric carbon atom. The same basic substances that catalyze racemization of acylated amino acids promote the formation of azlactones and it is reasoned that the acceleration of racemization is due to increased speed of formation of an intermediary azlactone and not to a direct effect on the configuration of the asymmetric carbon atom. Benzoyl chloride is more effective than acetic anhydride for azlactonization.

g. Hydantoin formation

Treatment of an amino acid with urea at elevated temperature leads to the formation of the corresponding hydantoic acid (163).

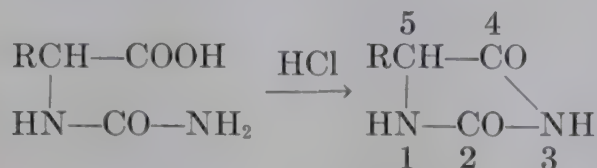


If the amino acid is optically active, use of urea always results in an optically inactive hydantoic acid. Optically active products,

however, may be obtained by treatment with potassium cyanate (59).



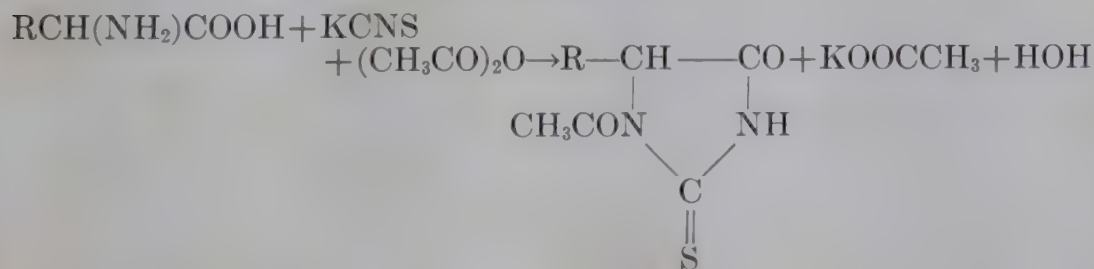
The hydantoic acids undergo ring closure on refluxing with strong hydrochloric acid.



Hydantoins are converted to the corresponding hydantoic acid by mild treatment with alkali while more vigorous treatment yields the parent amino acid.

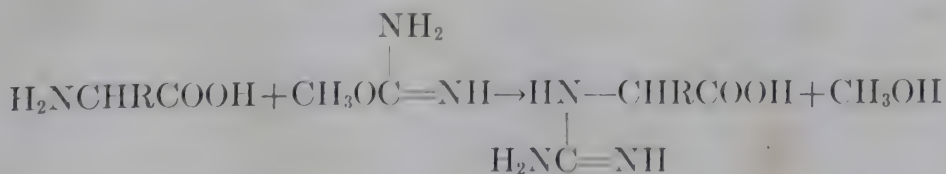
An optically active hydantoin is racemized in the cold by a trace of alkali (59). Such racemization does not occur with hydantoic acids nor with hydantoin lacking a hydrogen atom at the 5-position (36). It is argued, however, that enolization cannot be the only cause of racemization since substitutions at the 1 and 3 positions do not prevent loss of optical activity.

When amino acids are treated with potassium thiocyanate in the presence of acetic anhydride excellent yields of optically active acetylthiohydantoins are obtained (138).



h. Guanidino acids

In the presence of alkali the amino acids are converted to the guanidino acids by treatment with O-methylisourea, (141), S-alkylisothioureas, or cyanamide.



Glycocyamine (guanidoacetic acid) has been prepared in excellent yield from glycine and S-ethylisothiurea (38).

i. Reactions with carbonyl compounds

Sørensen (216) used formaldehyde to suppress the basic properties of the amino acids and thus enable him to titrate them as strong acids. For the nature of the reaction of formaldehyde with amino acids, see Chapter VII.

Formation of the benzyldine derivative of arginine (39) is the basis of a preparative method for this amino acid from a protein hydrolysate.

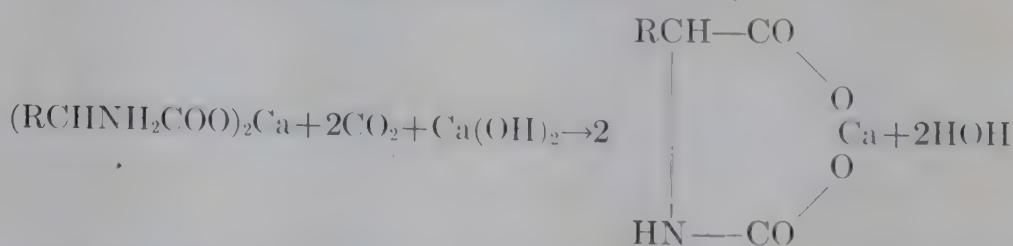
j. Salt formation

Amino acids being amphoteric in nature form stable salts with both acids and bases. Some of these normal salts, especially those of the sulfonic acids, are sparingly soluble and have proved of value in the isolation and purification of a number of the amino acids (Chap. IV).

In addition to the ionic type of salt formation, amino acids form complex salts with certain of the heavy metals. Some of these complexes are insoluble and have been used in separation procedures (41). The heavy metals most commonly used are silver, mercury, lead, zinc and copper. The insolubility of these salts is apparently due to the formation of high molecular weight complexes involving the secondary valences of the metals.

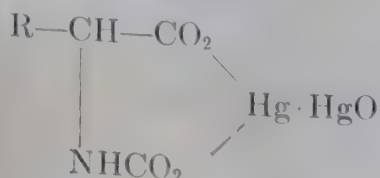
k. Carbamino acid formation

In alkaline solution the amino acids react with carbon dioxide to form carbamino acids whose calcium and barium salts are stable and will precipitate from dilute ethanol (214).



These salts decompose in boiling water to the free amino acid and the metal carbonate.

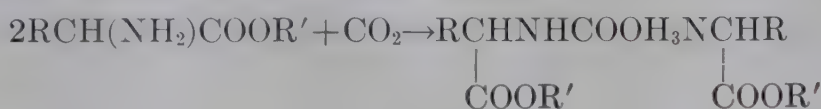
Neuberg and Kreh (176) have used the insolubility of the basic mercury salts of the carbamino acids as the basis for a method of group precipitation of the amino acids and many of the peptides. The salts are of the general formula:



The salts of valine and proline are incompletely precipitated. The original substance is readily recovered by decomposition with hydrogen sulfide.

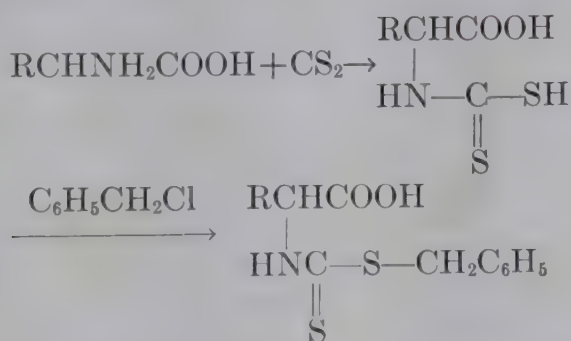
Carbamino salts because of their lability are believed to play an important part in the transport of carbon dioxide by the blood (197).

When the esters of amino acids are dissolved in ether and treated with carbon dioxide crystalline compounds are obtained which are stable below 0° and at room temperature in an atmosphere of carbon dioxide (110). These compounds have been identified as salts of one molecule of the free N-carboxy- α -amino acid ester with one molecule of the corresponding α -amino ester



Carbon dioxide is quantitatively evolved in the presence of strong acids.

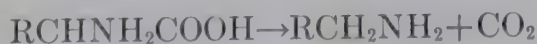
A reaction analogous to that with carbon dioxide occurs with carbon disulfide (215). Amino acids when treated with carbon disulfide in aqueous potassium hydroxide react at room temperature to form the corresponding dithiocarboxylic acid which may be isolated as the benzyl ester.



Aspartic and glutamic acids, unlike asparagine and glutamine, do not form insoluble dithio esters.

2. Irreversible Reactions

a. Decarboxylation



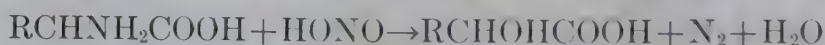
The loss of carbon dioxide by an amino acid to yield the corresponding organic amine may be effected by a variety of methods. The early investigators in certain cases obtained good yields by heating the amino acid to a high temperature in the dry state. This method is not applicable to large scale work. Excellent results

have been obtained by heating in a high boiling medium. Glycerol and glycol have been used but these substances have such an avidity for water that they favor the formation of diketopiperazines. Johnson and Daschavsky (137) obtained tyramine from tyrosine in 95 to 97% yield by heating to 260–5° in a mixture of diphenylmethane and diphenylamine while Tutiya (227) has reported a 57% yield for the decarboxylation of methionine in liquid paraffin at 250°. Others have reported desirable results with catalytic amounts of barium, iron, or copper.

The amino acids are also decarboxylated by a variety of putrefactive organisms. Recent evidence indicates that there exists specific decarboxylases for amino acids and in this connection Gale and Epps (115) have made enzyme preparations which are specific for lysine and tyrosine (see Chap. II).

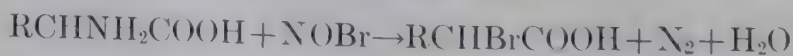
b. Deamination

1. **Nitrous acid.** When α -amino acids are treated with nitrous acid in the cold the nitrogen of the amino group is converted quantitatively to molecular nitrogen and the α -hydroxy acid is formed.



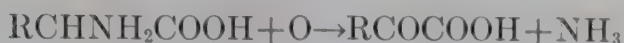
Van Slyke (228) has developed an extremely useful and precise method of determining α -amino nitrogen by measuring the nitrogen evolved in this reaction. This method will be discussed in more detail in Chapter II.

2. **Nitrosyl halide.** A variation of the above procedure is to treat the amino acid with nitrosyl chloride or bromide whereupon it is deaminated but the hydroxy acid so produced is immediately converted to the corresponding α -halogen acid.



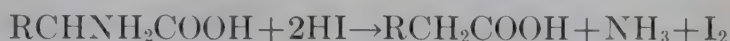
Although this reaction has been considered general for all amino acids, Zemplen and Csürös (260) report that it can be applied only to α - and β -amino acids and that the yields of bromo acids from the latter are low. Fischer (94) and others have used it to establish the configurational relationships of amino acids. It is of interest that the optically active ester of an amino acid subjected to this reaction yields upon hydrolysis an α -bromo acid of opposite rotation from that obtained from the corresponding optically active amino acid.

3. **Enzymatic deamination.** When amino acids are deaminated in the animal body they are also simultaneously oxidized.



This reaction was shown by Krebs (150) to take place under the influence of the enzymes of kidney slices with both the L- and D-amino acids indicating the presence of two deaminating systems in this organ.

4. **Hydriodic acid.** Amino acids, when heated to 200° with hydriodic acid are deaminated to the unsubstituted acids (133).



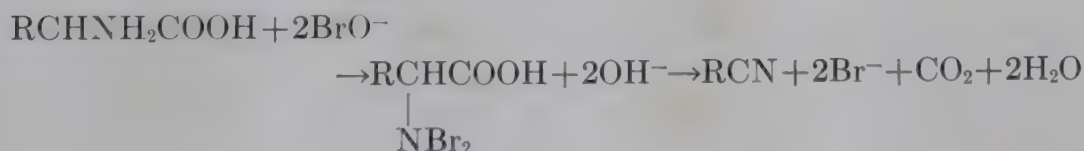
c. Oxidation

Apart from the biological oxidative deaminations discussed in 2b3 which leaves the carboxyl group intact there are several oxidative reactions which involve both the amino and carboxyl groups with or without the evolution of ammonia or carbon dioxide or both.

1. **Hypohalites.** Amino acids and their esters when treated with sodium hypochlorite form the N-chloro derivatives which are unstable and upon heating rapidly decompose to aldehydes with the evolution of carbon dioxide and ammonia (153).



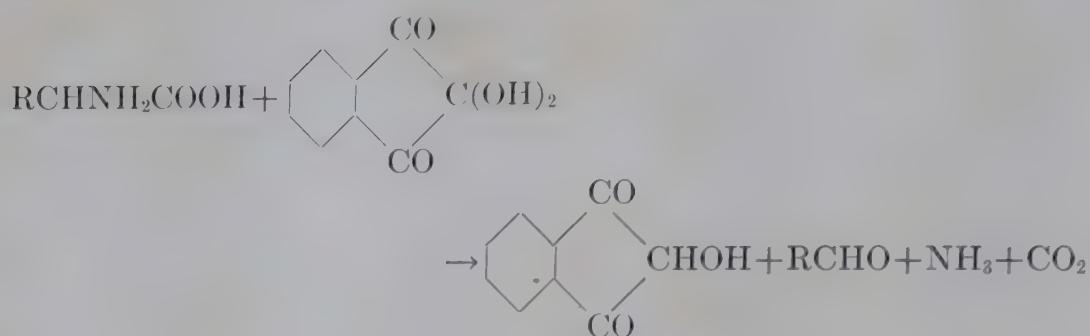
This reaction also occurs with sodium hypobromite but, under conditions which suppress alkalinity, a second reaction involving nitrile formation is favored (113).



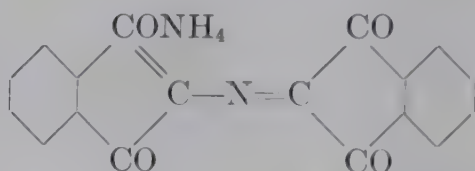
2. **Chloramine T.** Dakin and collaborators (62) report that one mole of chloramine T or *p*-toluenesulfonchloramide per mole of amino acid will cause the formation of aldehydes, carbon dioxide, and ammonia, as does sodium hypochlorite, but two moles of these reagents, like sodium hypobromite (61), will cause the production of nitriles.

3. **Carbonyl compounds.** The reaction of the amino acids with ninhydrin, triketohydrindene hydrate, is used as the basis of a qualitative color test for detecting α -amino acids and of a precise quantitative determination by measurement of the carbon dioxide or ammonia liberated (230).²

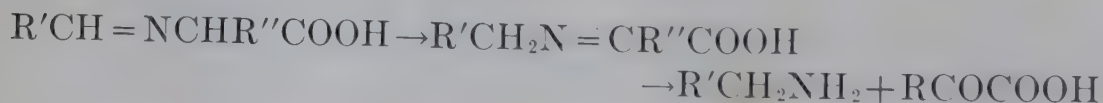
² This reaction will be treated in more detail in Chapter II. A similar reaction occurs with isatin.



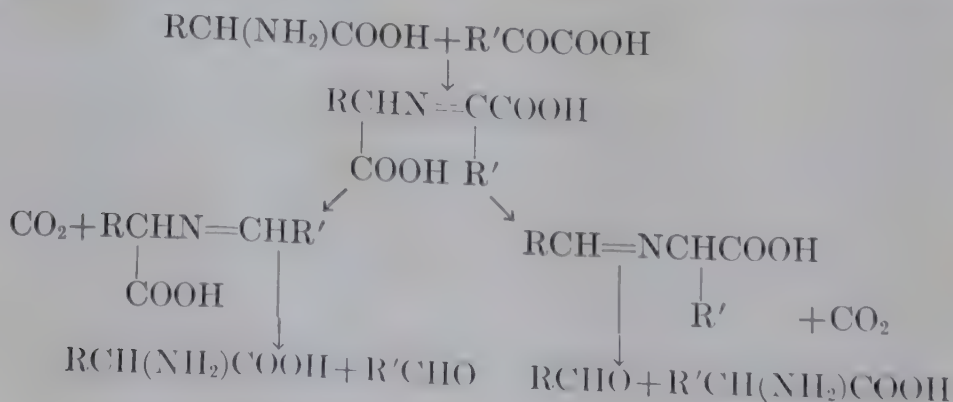
The ammonia formed reacts with triketohydrindene and its reduction product to yield the characteristic blue condensation product.



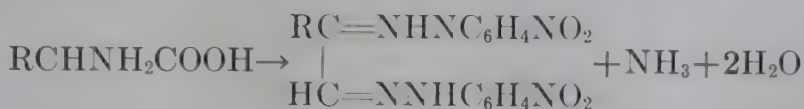
When amino acids react in the cold with formaldehyde and other aldehydes they form compounds from which the amino acid may be recovered by hydrolysis. However, when they are heated with any of a large number of carbonyl compounds including aldehydes, sugars and α -keto acids they undergo decomposition of the following type:



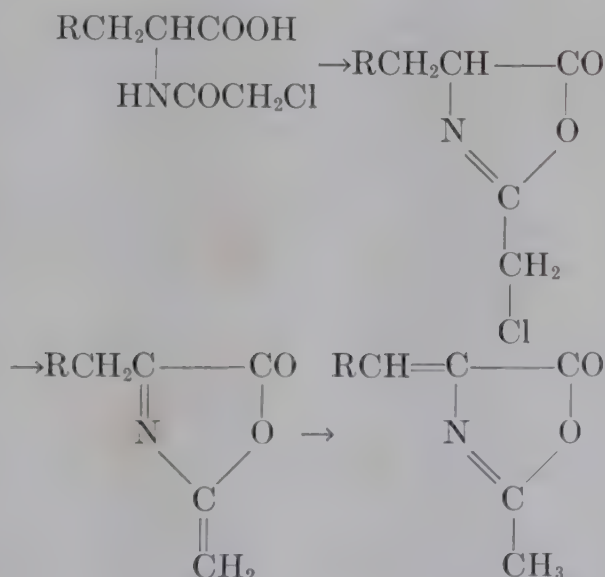
The reactions with α -keto acids have their counterpart in the living cell where α -keto acids are converted to amino acids by reaction with another amino acid under the influence of the transamination enzyme systems (40). In the *in vivo* reaction, however, one of the reactants must be a dicarboxylic acid. The reaction *in vitro* has been shown by Herbst (127, 129) to involve decarboxylation simultaneously with the rearrangement and to proceed in two directions.



4. ***p*-Nitrophenylhydrazine.** An extraordinary reaction involving both oxidation and reduction of the amino acid molecule is that with *p*-nitrophenylhydrazine (60). The bisnitrophenylhydrazone of the corresponding α -ketoaldehyde is formed with the evolution of ammonia.



5. **Action of acetic anhydride on chloroacetyl amino acids.** When chloroacetyl amino acids are warmed with acetic anhydride azlactonization occurs, hydrogen chloride is evolved followed by rearrangement so that the final dehydrogenation product is the unsaturated methyl azlactone (27).



6. **Other oxidations.** With a lead anode in dilute sulfuric acid solution amino acids are oxidized electrolytically at 100° to the next lower aldehyde and acid, while with platinum electrodes in nitric acid solution most of the amino acids yield oxalic acid (220). Exposure to ultraviolet light results in oxidation with the elimination of ammonia (174). In most cases the next lower aldehyde is one of the products of the reaction. Other oxidizing agents which cause degradation of the amino acid to the next lower aldehyde are peroxides (58), persulfates (152), oxygen in the presence of charcoal, palladium black (249), iron (1, 122), ozone and silver oxide (128).

Toennies and Homiller (223) have made a detailed study of the oxygen consumption of the amino acids when treated with hydro-

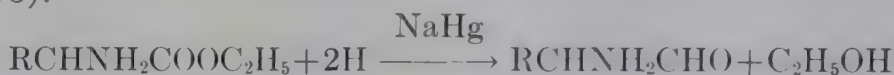
gen peroxide in formic acid. The amount absorbed varies from zero with isoleucine, leucine, and phenylalanine to five atoms per molecule with cystine.

d. Reduction

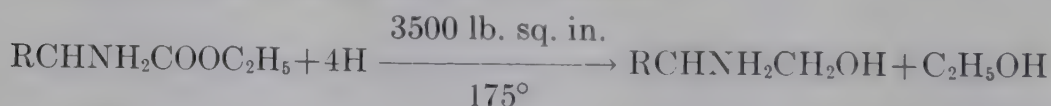
Reference has been made to the reductive deamination of amino acids by hydriodic acid (p. 39). This same reaction may be carried out with gaseous hydrogen by a suspension of the microorganism, *Clostridium sporogenes* (131).

In general the amino acids themselves are not affected by reducing agents. Their esters and anhydrides may, however, be reduced in a variety of ways.

1. Reduction of ester to aldehyde. The esters of the amino acids are reduced by sodium amalgam to the corresponding aldehyde (95, 175).

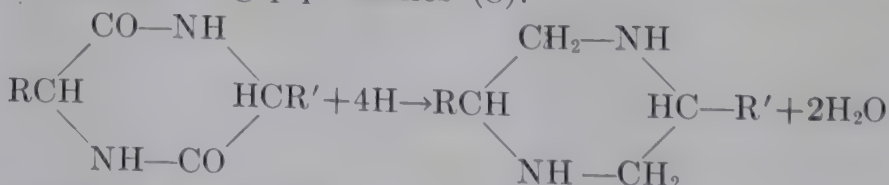


2. Reduction of ester to alcohol. The esters of the amino acids may be reduced with gaseous hydrogen at high pressures in the presence of copper chromite catalyst (52). If dioxane is the solvent the unsubstituted alcamines are obtained.



The N-dialkyl derivatives are prepared by the same procedure with methyl or ethyl alcohol as solvent. Higher alcohols tend to yield the N-monosubstituted derivatives although the N-disubstituted compounds can sometimes be obtained by use of more drastic conditions of reduction.

3. Reduction of diketopiperazine. By use of sodium and amyl alcohol, sodium and ethanol, zinc and acetic acid or zinc and hydrochloric acid the diketopeperazines of the amino acids are reduced to the corresponding piperazines (8).



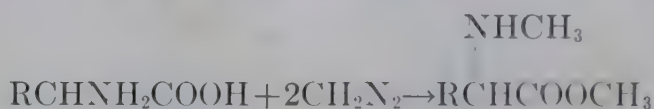
e. Reaction with acetic anhydride and pyridine

When amino acids are heated to 80–90° with a mixture of acetic anhydride and pyridine, carbon dioxide is evolved and two acetyl groups are introduced into the molecule (65, 155).

More recently Sanger (200) has observed that this reaction will proceed at room temperature with N-2,4-dinitrofluorobenzene. This reagent will not react with aliphatic hydroxyl groups but does attack the phenolic OH of tyrosine, the imidazole ring of histidine, and the sulfhydryl group of cysteine to yield the di-2,4-dinitro-phenyl derivatives of these amino acids.

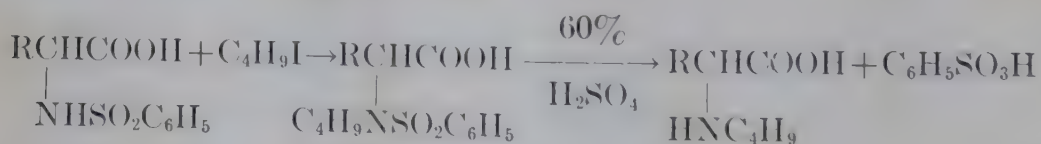
This reaction has been of value in characterizing the free amino groups in a protein. The protein is alkylated and hydrolyzed, after which the alkylamino acids are separated by chromatography and identified.

3. Action of diazomethane. When treated with diazomethane, all of the amino acids tested, with the exception of glycine and alanine, which are recovered unchanged, are substituted on both the carboxyl and amino groups according to the following reaction (130):



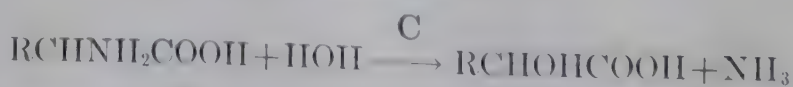
✓ **4. Action of sulfurous esters.** When amino acids are treated with dialkyl sulfites, a portion of the sulfurous ester rearranges to the sulfonate which forms a salt with the alkylated amino acid (241). Thus glycine and dimethylsulfite yield trimethyl (carbomethoxy methyl) ammonium methane sulfonate, $(\text{CH}_3\text{SO}_3)^- [\text{N}(\text{CH}_3)_3\text{CH}_2\text{COOCH}_3]^+$.

5. Alkylation of sulfonated amino acids. If the benzenesulfonyl derivatives of the amino acids are treated with alkyl halides whose carbon chain length is not greater than five, the amino acid is alkylated and the N-monoalkyl derivative may be obtained by treatment with sulfuric acid (55).



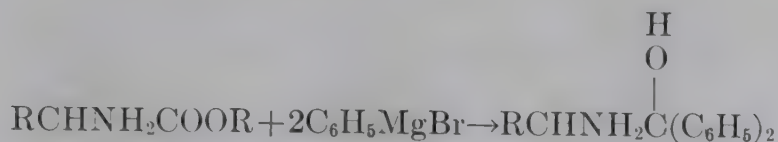
g. Hydrolysis

Certain of the amino acids undergo slow hydrolysis in the presence of bone black (258). Glycolic acid from glycine has not been isolated but lactic acid from alanine and malic acid from aspartic acid have been isolated and identified.



h. Grignard reaction

The esters of the amino acids form addition compounds with Grignard reagents which when decomposed yield amino alcohols (31, 259).



Grignard reagents have been used to determine the identity of the amino acid residue with the free carboxyl group in a peptide chain. After the Grignard treatment the peptide is hydrolyzed with acid, whereupon the amino alcohol is converted to a ketone and ammonia.

3. Reactions of Hydroxyamino Acids

a. Reduction

The hydroxyl groups of all of the hydroxyamino acids may be reduced with red phosphorus and hydriodic acid to the corresponding aliphatic derivative (32).

b. Esterification of hydroxyl group

Sakami and Toennies (198) have described a method for the differential acetylation of the hydroxyl groups of hydroxyamino acids in the presence of perchloric acid and have also reviewed the literature which indicates that in neutral or acid solution the esterification of the hydroxy group is favored, while in basic solution acylation of the amino group takes place preferentially. Conversely, alkaline treatment cleaves the O-acyl group without affecting the N-acyl bond, while acid hydrolysis more readily splits the N-acyl linkage.

The hydroxyamino acids may be phosphorylated (188) and such compounds are of biological significance since phosphopeptides in which the phosphoric acid is attached by ester linkage to hydroxyamino acids have been isolated (162) from casein.

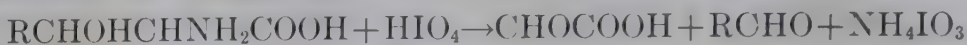
It is now known that the gel-like substances obtained by the treatment of proteins with cold concentrated sulfuric acid are the resultant products of esterification of the free hydroxyl groups of the proteins (192). The acid sulfates of serine, threonine and tyro-

sine have been prepared directly from these amino acids. In this respect, cysteine reacts as a hydroxyamino acid.



c. Oxidation

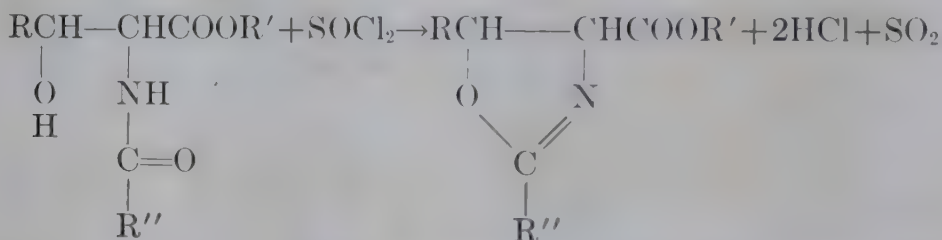
With periodic acid (177) or lead tetraacetate (34) the amino acids which possess an hydroxyl in the β -position are oxidized quantitatively to glyoxylic acid and the corresponding aldehyde.



This reaction has been used for the quantitative estimation of the β -hydroxyamino acids, the aldehyde formation being estimated by gravimetric or colorimetric procedures.

d. Oxazoline formation

N-Acylated β -hydroxyamino acid esters, when treated with a dehydrating agent, lose a molecule of water and form an oxazoline derivative (26).



As might be expected from the discussion in part b of this section, mild acid hydrolysis of the oxazoline compound results in the formation of the O-acyl amino acid ester and if this latter derivative is then treated with an excess of alkali the acyl radical migrates to the amino group.

Pfister *et al.* (182) have observed that in the process of forming and opening the oxazoline ring a Walden inversion takes place so that an optically active compound is converted into one of its diastereoisomers. It is believed but not confirmed that the inversion takes place at the β -carbon and occurs during the ring closure step. This inversion has been used in an excellent synthesis in which the readily available DL-allothreonine is smoothly converted to DL-threonine.

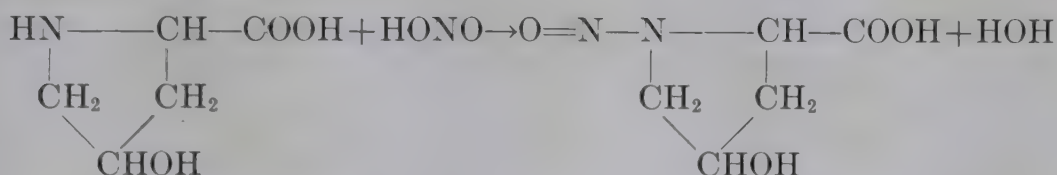
4. Reactions of Proline and Hydroxyproline

Because proline and hydroxyproline are derivatives of pyrrolidine

they often undergo reactions quite different from those of the primary amino acids.

a. Effect of nitrous acid

Nitrogen is not released by the action of nitrous acid nor is the ring opened. Dakin (64) attempted to take advantage of this difference in reactivity for the separation of proline and hydroxyproline from a gelatin hydrolysate. He found, however, that an excess of nitrous acid reacted with hydroxyproline, presumably to form the N-nitroso derivative.



This has been substantiated in the author's laboratory where it was observed that treatment of hydroxyproline with nitrous acid yielded a clear non-crystallizable syrup completely soluble in ethanol from which hydroxyproline could be recovered in fifty per cent yield by catalytic reduction.

b. Reaction with acetic anhydride and pyridine

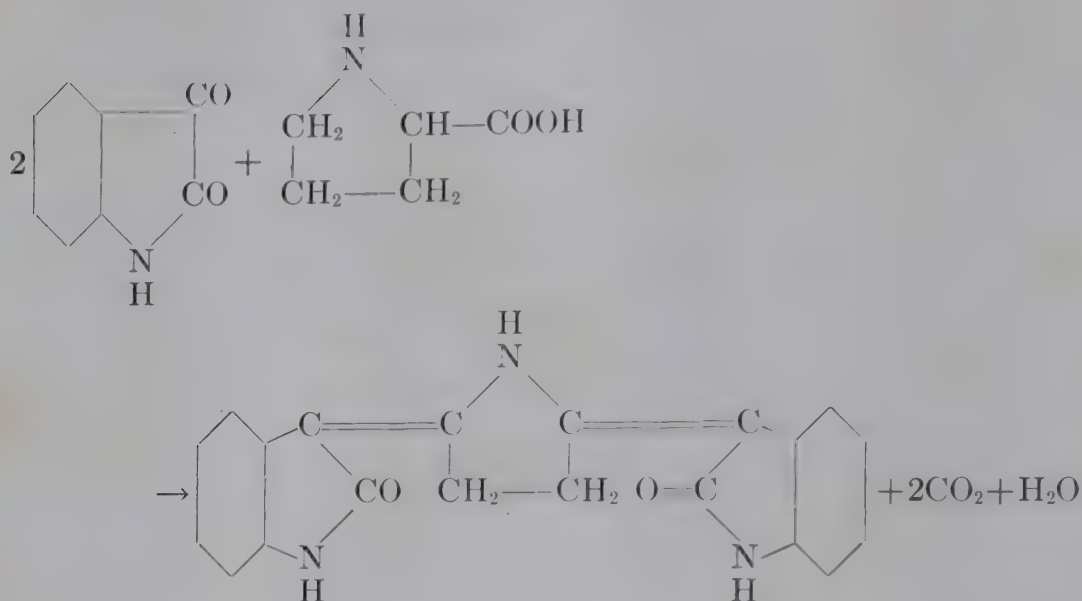
Unlike the primary amino acids, (p. 42) proline and hydroxyproline, when treated with acetic anhydride in the presence of pyridine, are only acetylated and do not evolve carbon dioxide with the production of an acetamidomethyl ketone.

c. Azlactone formation

Because of the secondary nature of the amine group of proline and hydroxyproline, these amino acids do not form azlactones when acylated and treated with a dehydrating agent.

d. Reaction with carbonyl compounds

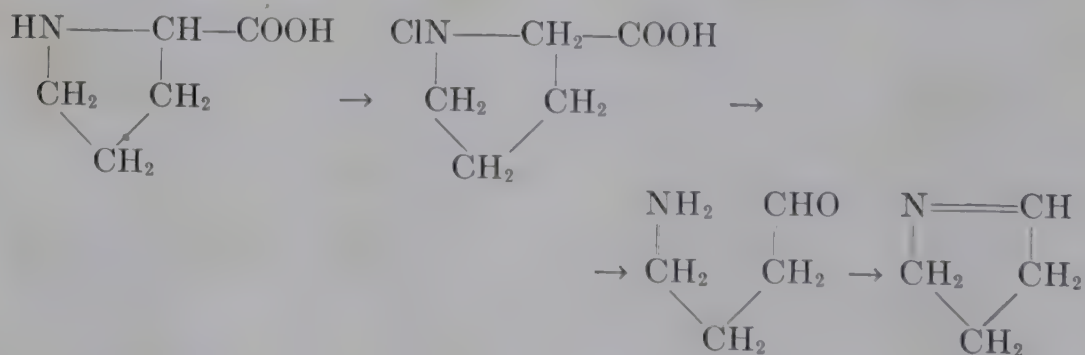
Proline and hydroxyproline obviously cannot form Schiff bases or dimethylol compounds. Apparently they do, however, form monomethylol derivatives, for in an excess of formaldehyde, their basic dissociation is suppressed sufficiently that the carboxyl group may be titrated with standard alkali. They also undergo decarboxylation and condensation with such carbonyl compounds as isatin and ninhydrin (119). The reaction with isatin and proline is as follows:



The condensation products formed are bright blue in color and have been used for the identification and estimation of proline and hydroxyproline.

e. Oxidation

Oxidation of proline with sodium hypochlorite opens the ring with the formation of an amino aldehyde which loses water to form pyrroline (153).



f. Reduction

By the action of putrefactive bacteria proline and hydroxyproline both are converted into δ -aminovaleric acid, $\text{H}_2\text{N}-(\text{CH}_2)_4\text{COOH}$ (142).

VI. IDENTIFICATION AND PURITY DETERMINATION OF THE AMINO ACIDS

Elementary analysis, formol titration and, with optically active amino acids, specific rotation have been used to demonstrate the

purity of crystalline amino acids. None of these methods give more than an indication of purity since there is always the possibility of contamination with an isomer, with an acid of the same titration value, or with a compound of identical optical rotation.

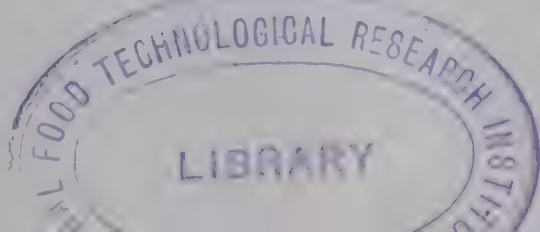
There remains one characteristic property which when accurately determined under proper conditions not only serves to identify the amino acid but also will show its degree of purity. This is its solubility. Phase purity determinations may be applied to the amino acids (226) with accuracy, the experimental error in most cases being considerably less than one per cent. This method, in its application to the purity of proteins, is discussed in detail in Chapter VIII. The solubility diagram is obtained by plotting the solubility against the weight of solute used per unit weight of solvent under rigidly controlled environmental conditions. If the compound is pure the curve consists of two intersecting straight lines one with a slope of 45° until saturation is reached and after saturation a straight line parallel to the abscissa (see Fig. 16, Chap. VIII). If impurities are present, the curve will have definite breaks at the points at which the solvent becomes successively saturated with these components (see Fig. 17, Chap. VIII). The solubilities of the various components may be calculated from data obtained by extrapolation of the segments of the curve to the ordinate (Fig. 17, Chap. VIII). The composition of the solute may be calculated from the slope of the curves. In this manner it was determined that DL-isoleucine prepared from secondary butyl bromide by the malonic ester synthesis contains approximately 8% of one minor component and 4% of a second. From such data the possibility of purification without exorbitant loss of material by recrystallization or equilibration with the solvent is indicated. The method fails only if the substance studied is unstable in solution, if the composition ratios of a mixture of substances are identical with their solubility ratios, or if no suitable solvent can be obtained. With the amino acids mixtures of ethanol and water have been used to advantage.

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Chapter II

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I. INTRODUCTION

INVESTIGATIONS of the composition of proteins may be classified as follows: 1. *Identification of the amino acids*. This first phase may be considered fairly complete. If proteins contain any unidentified amino acids, they are probably present only in minor quantities (cf. 50). 2. *Quantitative analysis of the amino acids*. During the past decade there have been important, almost revolutionary, advances in amino acid analytical methods. These developments will be surveyed in this chapter. 3. *Relative position of the amino acid in the peptide chain*. Studies of this type will depend upon identification and characterization of partial hydrolysis products (259) and of end groups (93). Work in this field is still in its early stages and will not be discussed in detail in this book.

The vast amount of analytical work on amino acids that preceded the evolution of the more accurate and convenient quantitative methods has been summarized by Block and Bolling (27). Although some of the information so painstakingly accumulated is now proving useful, much of the data will require correction when the proteins in question are re-analyzed by the newer methods.¹

1. "Primary Standard" and "Routine" Methods

Stein (248) has emphasized the necessity of having available two kinds of amino acid analytical techniques, the "primary standard" and the "routine." A primary standard method should give data of the highest accuracy possible, with which data obtained by other methods might be compared. It need not necessarily be a convenient or rapid method, nor adaptable to the handling of small quantities of protein. At the present writing, "isotope dilution" methods appear to approach most closely the criteria for primary standards. However, several others have been so carefully worked out and controlled that they may be assumed to be capable of supplying standard data as well.

Routine methods would be accepted or rejected on the basis of comparative results with data obtained with the standard methods. The routine methods should be rapid, convenient, inexpensive, and adaptable to microanalytical techniques. Most of the proposed methods for amino acids to be discussed in this chapter will fall into the latter class.

¹ Reference will be made only to the most recent or pertinent papers in discussing the various analytical methods. The reader should consult the comprehensive literature surveys and bibliographies prepared by Block and Bolling (27) and Martin and Synge (192) for information concerning their historical development.

In future amino acid analytical work, it would be desirable to have available two or more "standard" proteins, the amino acid content of which would have been carefully worked out by "primary standard" methods. Investigators wishing to determine the accuracy of new methods could compare their results with those available for the standard protein, *on a sample of the standard protein*. At the present time, when differing analytical values are obtained for the same protein (as has been the case with crystalline insulin (33, 50)), it is not possible to know how much of the variation may be assigned to differences in the protein preparations used, and how much to differences in analytical techniques.

2. General Analytical Technique

If possible, protein samples should be kept in constant temperature, constant humidity rooms. After the samples have come to equilibrium, portions can be taken for moisture and nitrogen determinations. For the determination of moisture, drying at 100–110° *in vacuo* (cf. 38) to constant weight gives usable data. Bull (42) recommends 24 hours at 105° *in vacuo*. The determination of nitrogen in proteins by the Kjeldahl procedure is not simple and has been discussed in detail by Chibnall, Rees and Williams (51), Kirk (160), and Hiller, *et al.*, (136a). The lengthy digestion times suggested by some investigators have been found not to be necessary by White and Secor (292, 293, cf. 131a), who use a microtechnique and the Clark procedure (55) in which mercury is the catalyst.

If the protein sample to be analyzed is not in equilibrium with an atmosphere of constant humidity at the time of taking samples, aliquots should be weighed out *at the same time* for separate determinations of nitrogen and moisture. Many dried proteins are extremely hygroscopic, and it is therefore difficult to weigh out samples accurately after they have been dried.

Most investigators report amino acid analytical data on a moisture- and ash-free basis. Ash is usually determined by heating a sample of the protein to constant weight in a muffle furnace at 550–600°. Residual ash in proteins prepared by the classical procedures usually represented inorganic salts which could not be washed out of the precipitates. It would not seem to be necessary to correct for ash in thoroughly electrodyalyzed protein preparations. In many cases the exact interpretation of an ash determination is difficult and, indeed, the residue may well represent an integral part of the protein as it does in the case of hemoglobin or

the phosphoproteins (cf. 289, 79). Corrections for ash *and* sulfate, reported by some investigators, may be redundant. If analytical values for amino acids are reported as per cent of total nitrogen, these determinations and corrections are unnecessary (see p. 104).

3. Methods of Hydrolysis

For most amino acid methods, proteins must be hydrolyzed prior to analysis. The commonest technique is to reflux the protein with five to 20 times its weight of approximately 20% (6*N*) hydrochloric acid for 24 hours, although some amino acids are liberated quantitatively after considerably shorter hydrolysis periods (four to eight hours). If necessary, most of the excess hydrochloric acid can be removed by distillation to dryness *in vacuo*, repeated several times after the addition of water. Numerous variations of conditions are possible. Lower concentrations of acid require heating at higher temperatures or for longer periods of time (cf. 228). Where an amino acid is sensitive to long-continued exposure to hot acid, a time series can be run. For example, Hess and Sullivan (133) showed that maximal cystine values were obtained after six hours of hydrolysis. Hydrolysis by acid in 20% urea solution (36) has been proposed to minimize humin formation. Sulfuric acid (8*N*) has often been used where complete removal of inorganic anions is desired, since the sulfate ions can be quantitatively precipitated with barium hydroxide. However, it is difficult to wash out absorbed amino acids, particularly aspartic acid and cystine, from the barium sulfate precipitate (52).

Hydrolysis with hydriodic acid finds special use in a method for the determination of methionine and cystine (12, 37, 176). A mixture of constant boiling formic acid and concentrated hydrochloric acid (1:1) gave maximal values for the cystine content of insulin (194).

Proteins are also readily hydrolyzed by refluxing or autoclaving with 5*N* sodium or barium hydroxide for six to 24 hours. Barium hydroxide hydrolysis is more rapid and more complete (279, 288) and appears to be less destructive for tryptophan (236). Alkaline hydrolysis is recommended for amino acids that are labile under the conditions of acid hydrolysis (tryptophan, and tyrosine in the presence of carbohydrates (cf. 175, 164, 117)).

Enzymatic hydrolysis so far has found only limited use, since it has not yet been possible to find the proper combinations of enzymes that will hydrolyze a protein completely to its constituent

amino acids. Nevertheless, several investigators have used partial hydrolysis by enzymes in the determination of tryptophan, methionine, and arginine by microbiological and colorimetric methods. Enzymatic hydrolysis of proteins has been found to give more significant values in the determination of total tryptophan plus tyrosine by the Folin procedure (95).

4. Destruction of Amino Acids During Hydrolysis

One of the serious unsolved problems in the analytical chemistry of proteins concerns the extent of destruction of amino acids during the hydrolytic procedure. It is not difficult to determine the rate of destruction or loss of an individual amino acid when it is subjected to the hydrolytic conditions with or without other amino acids. But these rates do not necessarily reflect the rate of destruction of the amino acid while it is in peptide linkage, and, in many reactions, the amino acid in peptide-linkage is more reactive than after its liberation.

Furthermore, rates of destruction depend upon the presence or absence of other constituents, such as carbohydrates (178, 70a), either in the protein or in the hydrolysis mixture. The interaction of amino acids upon each other has not been generally appreciated. For example, cystine is reduced to cysteine by reaction with tryptophan during acid hydrolysis, and cysteine can react with the pyruvic acid present as the result of degradative changes in serine (207). The possibility of minimizing some of these reactions by keeping the hydrolyzing solution as *dilute* as possible with respect to its content of amino acids, etc., is worthy of consideration.

Evidence for absence of destruction during hydrolysis should include data showing that: 1. the conditions of hydrolysis do not cause destruction of the amino acid alone, or in the presence of a mixture of other amino acids simulating the protein hydrolysate, or in the presence of the protein hydrolysate; 2. if the amino acid is added before hydrolysis, it can be quantitatively recovered after hydrolysis; 3. amino acid analyses of the protein do not differ when two or more different methods of hydrolysis are used; and, 4. where possible, analyses on the intact protein agree with those obtained in hydrolysates. In some cases, independent analytical methods may be brought to bear on the problem. For example, the total methionine, cystine and cysteine should account for the non-sulfate sulfur content of the protein (cf. 33).

There is reliable evidence that the basic amino acids (lysine, arginine, histidine) are quite stable under the conditions necessary

for complete hydrolysis of proteins by acids (228) although slight but detectable destruction of histidine has been shown to occur in one instance (263). On the other hand, tryptophan, serine, threonine, cysteine, tyrosine, and phenylalanine are subject to considerable destruction under these conditions. The exact fate of many of the other amino acids is not known, but it is quite possible that an amino acid may be stable during the hydrolysis of one protein and labile in the next.

Alkaline hydrolysis is particularly destructive for cystine and arginine but also causes more or less general deamination of amino groups (290). Decarboxylation of glutamic acid to α -aminobutyric acid can be accomplished by boiling 20% sodium hydroxide (1). Tryptophan and tyrosine have often been determined in alkaline hydrolysates. Kuiken, Lyman and Hale (164) claim that destruction of tryptophan during alkaline hydrolysis (117) is due to oxidation and can be prevented by the addition of cysteine.

5. Racemization

Wherever methods that differentiate between the L- and the D-amino acids are to be used, the possibility of racemization during hydrolysis must be considered. In general, free amino acids are not rapidly racemized by boiling with acids. In a careful study, Graff, Rittenberg and Foster (110) showed that tissue proteins contained less than 1% racemic glutamic acid after being boiled with 20% hydrochloric acid for 24 hours. However, other investigators have indicated that up to 6% racemization of free L-glutamic acid may occur under the same conditions (e.g., 22, 297). Cysteine is completely racemized by boiling 5*N* hydrochloric acid in five days (139, 140).

As with hydrolytic destruction, the rate of racemization of amino acids in peptide linkage may be different than that after isolation (cf. 169), and it is possible that considerably more racemization occurs during hydrolysis than has been detected. Martin and Synge (190) isolated almost completely racemic phenylalanine from an acid hydrolysate of wool, although Foster (92) found that phenylalanine in an hydrolysate of horse hemoglobin was less than 2% racemized.

High temperatures cause increased racemization (31). Schein and Berg (228) obtained racemic arginine from a sample of casein that had been hydrolyzed with 25% sulfuric acid at 160° for 24 hours.

Racemization of the amino acids of proteins during alkaline

hydrolysis is rapid and considered to be complete after six hours of refluxing with 5*N* sodium or barium hydroxide (cf. 168). Isolated amino acids are racemized much more slowly. Tryptophan and tyrosine values obtained by microbiological techniques after alkaline hydrolysis are multiplied by two to correct for the amount of inactive D-amino acids present. Here too it may be necessary to emphasize the possibility that all amino acids may not be completely racemized during such treatment, since, for example, Dakin and Dudley (66) isolated amino acids of differing degrees of racemization from alkali-treated casein.

It is usually assumed that all amino acids in intact proteins are of the L-configuration, and that D-amino acids present in hydrolysates are products of racemization. However, D-amino acids have been identified as normal constituents of several bacterial products (gramicidin, tyrocidine, etc., cf. 192) and the possible occurrence of small amounts of D-amino acids in proteins cannot be entirely dismissed. Chibnall and coworkers (50) detected more D-glutamic acid in gliadin and lactoglobulin than might have been expected from racemization only. Weil and Kuhn (291) reported that the leucine fraction of a sample of old horse hair contained 3.3% D-leucine, whereas none could be detected in similar fractions from young horse hair or casein.

Several of the methods to be described determine L-amino acids only. To the extent that they fail to detect the D-forms occurring naturally or formed during hydrolysis, they will yield erroneous results.

II. GENERAL METHODS APPLICABLE TO SEVERAL AMINO ACIDS

1. Isotope Dilution Method

This method (237) is based upon the principle that the usual laboratory procedures for isolating amino acids do not separate isotope-containing molecules from their normal analogues. If a known amount of an amino acid, that has been labeled by the incorporation of a stable or radioactive isotopic element, is added to a protein hydrolysate, and then the same amino acid is isolated from the mixture and purified, a determination of the concentration of isotope in the sample will give a direct measure of the amount of the amino acid originally present in the hydrolysate. The decrease in the concentration of labeled element from that present in the added amino acid to that present in the isolated

amino acid indicates the extent to which the labeled amino acid has been *diluted*. The equation used in the calculations is $B = (C_0/C - 1)A$, where B is the amount of amino acid originally present, A is the amount of labeled amino acid added, and C_0 and C are the concentrations of the labeled element in the added and isolated compounds, respectively. The technique has so far been applied only with amino acids labeled with N^{15} . The measurement of N^{15} requires the mass-spectrograph, an instrument which is not generally available.

Although the amino acid does not have to be isolated quantitatively, it is essential that it be absolutely pure. Numerous crystallizations and purifications are permissible, provided only that sufficient sample remains for the final determination. Carbon and nitrogen analyses alone are not sensitive enough to detect the presence of appreciable quantities of impurities. Final purity is established by the constancy of isotope concentration, specific rotation, and the melting points of the derivatives used, during successive stages of purification.

In most determinations (217, 110, 92, 237), the isotopic amino acid has been obtained by synthesis, and hence is in the racemic or DL-form. After addition to the protein hydrolysate, the naturally occurring L-form is isolated. Means for separating pure L from the DL-amino acid mixture require information concerning their relative solubilities. When the L-form is the more insoluble, purification is accomplished by recrystallization from sufficient solvent to keep the racemic form in solution.

The isotope dilution technique furnishes a method for determining the extent of racemization of an amino acid, since it is possible to estimate the extent of dilution of the unnatural isomer by a comparison of the isotope content of the isolated L-component with that of another fraction containing both isomers. The method of calculation will be found elsewhere (237, 110, 102), but, as an example, if no D-amino acid were present, the isotope concentration of the DL-amino acid after isolation should be one-half of that in the L-component plus one-half of that in the DL-component added. In the few experiments that have been reported (92, 110), the amounts of racemization encountered were too small to be significant. However, it is appreciated that the extent of racemization may vary not only from amino acid to amino acid but also with the mode of linkage of the amino acid in the protein molecule.

The amino acids that have been determined by the isotope dilution method are listed in Table I.

A modified method for determining amino acids by isotope dilution has recently been briefly described (158, 158a). The amino acids in a protein hydrolysate are reacted with a sample of *p*-iodophenylsulfonyl chloride (called PIPSYL chloride for conven-

TABLE I
ANALYTICAL METHODS AVAILABLE FOR THE INDIVIDUAL
AMINO ACIDS

	Isotope Dilution	Microbiological ^a	Solubility Product	Partition Chromatography	Colorimetry	Decarboxylase	Quantitative Isolation	Oxidation	Other
Alanine	+	+		+				+	Arginase - Aspartase
Arginine	+	+		+	+	+	+	+	
Aspartic acid	+	+		+			+	+	
Cysteine					+			+	Pyrrolidone carboxylic acid
Cystine		+			+			+	
Glutamic acid	+	+		+		+	+	+	
Glycine	+	+	+						
Histidine		+		+	+	+	+		
Hydroxyproline					+				
Hydroxylysine								+	
Isoleucine		+		+					
Leucine	+	+	+	+					
Lysine	+	+		+		+	+		
Methionine		+		+	+				
Phenylalanine	+	+		+	+				
Proline	+	+	+	+					
Serine		+						+	
Threonine		+						+	
Tryptophan		+			+				
Tyrosine	+	+		+	+	+			
Valine		+		+					

^a For further details, see Table II.

ience) in which the iodine (or sulfur) is radioactive. After removal of the excess reagent, an overwhelming excess, but accurately weighed amount, of the derivative of the same reagent, but *not* radioactive, with the particular amino acid to be determined, is added to the protein hydrolysate. The derivative is then reisolated and recrystallized to constant specific radioactivity. From its radioactivity, the extent of occurrence of the amino acid in the hydrolysate can be calculated (see also under Chromatography).

The advantages of this technique are: 1. The cheaper, more

abundant and more readily measurable isotopes can be used; 2. costly syntheses of amino acids containing isotopic carbon or nitrogen can be avoided; and, 3. reagents may be chosen from those most likely to give derivatives of high purity for each individual amino acid.

The isotope dilution method has the possibility of being the most accurate and reliable of any known, since the measurement of concentrations of stable or radioactive isotopes can be carried out with great precision.

2. Microbiological Methods*

(cf. 233, 244, 245)

The use of micro-organisms to assay amino acids was a natural development following the intensive study of their nutritional requirements for purposes of vitamin assays. The lactic acid bacteria, in particular, were found to require a number of different amino acids in order to obtain maximum growth (see Table II). When any one of the essential amino acids was omitted, growth failed. If the amino acid was restored, the amount of growth that occurred was in proportion to the amount of amino acid supplied.² Numerous amino acid analytical methods have been based on this principle.

One of the chief advantages of these methods is that they require only small amounts of material. A complete analysis of a protein requires less than 100 mg. Thus the amino acid composition of many rare proteins can be determined. In addition, the techniques are extremely simple and the time and expense involved is minimal. Many investigators are accumulating evidence to indicate that accuracy equal to that achieved with any other of the analytical methods is possible (cf. 219, 233, 70a).

* The suggestions of J. C. Lewis concerning microbiological techniques are gratefully acknowledged.

² Requirements of each organism for each amino acid do not appear to be absolute, but have varied in the hands of different investigators (see Table II). In his reviews covering microbiological amino acid assays, Snell (244, 245) has discussed the several reasons for this failure of agreement. Some of the chemical factors involved may be mentioned. For example, Stokes and Gunness (252) showed that in the presence of sufficient pyridoxamine, *L. delbrückii*, *L. arabinosus*, and *L. casei* no longer required lysine, threonine, and alanine. Lyman, *et al.* (182) found that the amount of carbon dioxide available to the organism was also a critical factor. In its presence, arginine, phenylalanine, and tyrosine were not essential for the growth of *L. arabinosus*. Stokes, *et al.* (255) demonstrated a close relationship between biotin and aspartic acid. With several organisms, aspartic acid was no longer required if biotin was supplied.

TABLE II

AMINO ACID REQUIREMENTS OF SOME LACTIC
ACID BACTERIA AND *Cl. perfringens*^a

Amino Acid	Microorganism ^b							
	LM	SF	LA	LB	LC	LD	LF	CP
Alanine	*	+	*	*	+ ²⁰	+	—	—
Arginine	+	+ ¹⁰	*	+	+	+	—	+
Aspartic acid	+ ¹	*	*	+	*	*	—	—
Cystine	+ ²	*	*	+	+	+	—	+
Glutamic acid	+	+	+ ¹⁸	+	+	+	+	+
Glycine	+ ³	*	—	+	+	—	—	—
Histidine	+ ⁴	+	—	+	+	—	*	+
Hydroxyproline	—	—	—	—	—	—	—	—
Isoleucine	+	+ ¹¹	+	+	+	+	+	+
Leucine	+	+ ¹²	+	+	+	+	+	+
Lysine	+ ⁵	+ ¹³	*	+	—	*	—	—
Methionine	+ ⁶	+ ¹⁴	+	+	*	—	+	+
Phenylalanine	+ ⁷	*	+	+	+	+	+	+
Proline	*	—	—	+ ¹⁹	—	—	—	—
Serine	+ ⁸	*	—	*	+	+	—	+
Threonine	+	+ ¹⁵	*	+	+	—	*	+
Tryptophan	+	+ ¹⁶	+	+	+	+	+	+
Tyrosine	+ ⁹	+	*	+	*	+	+	+
Valine	+	+ ¹⁷	+	+	+	+	+	+

^a The symbols have the following meanings: + indicates that the amino acid is essential; * has been used in those cases where the amino acid has been found to be essential by some investigators and not essential by others, or where high blanks but not necessarily maximum growth has been observed in the absence of the amino acid. In many such cases, conditions may be found suitable for the assay use of the organism. The symbols in bold face indicate the organism that has been used most often or is preferable for some other reason. — indicates that the absence of the amino acid from the medium is not detectable by decreases in growth. For the requirements of other lactic acid bacteria, see 70b, 77a, 83.

^b LM = *Leuconostoc mesenteroides* P-60 (ATCC 8042)

SF = *Streptococcus faecalis* R (ATCC 9790, 8043)

LA = *Lactobacillus arabinosus* 17-5 (ATCC 8014)

LB = *Lactobacillus brevis* (ATCC 8257)

LC = *Lactobacillus citrovorum* (ATCC 8081)

LD = *Lactobacillus delbrueckii* LD-5 (ATCC 9595), also probably *Lactobacillus casei* (ATCC 7469) (See Ref. 83)

LF = *Lactobacillus fermenti* 36 (ATCC 9398)

CP = *Clostridium perfringens* (Welchii) BP6K

The lactic acid organisms are obtainable under the numbers indicated from the American Type Culture Collection, Washington, D. C.

The numbered footnotes refer to publications containing details of assay procedure:

(1) 124, 224

(2) 131a, 215a, 244

(3) 161a, 235

(4) 76, 119, 147a

(5) 33, 75, 119, 145, 181, 224

(6) 73, 181, 215, 224

(7) 80

(8) 193a; 224

(9) 224

(10) 119, 147b, 224, 254

(11) 254

(12) 254

(13) 119, 181, 224, 254

(14) 181, 215, 254

(15) 116, 137, 146, 224, 254

(16) 164, 224, 254

(17) 119, 147, 254

(18) 71, 125, 170, 179

(19) 77a, 83

(20) 224a, 247c

The following references include assays not referred to above: 17, 19, 20, 32a, 39a, 70a, 81, 82, 115, 117, 120, 136, 137, 144, 147c, 161, 165, 180, 185, 232, 253, 300.

An example of a medium suitable for the assay of several amino acids is given in Table III. Each of the constituents is supplied in an amount which is in excess of that required for maximal growth. For the assay of any specified amino acid, this or some similar mixture, lacking only the amino acid in question, is the basic medium.³ Portions of the solution are introduced into a number

³ The purity of the amino acids used is important. L-tyrosine from natural

TABLE III
REPRESENTATIVE MEDIUM FOR MICROBIOLOGICAL
AMINO ACID ASSAYS^a

Constituent	mg. per liter	Constituent	mg. per liter
Amino Acids		MgSO ₄ · 7H ₂ O	800
DL-Alanine	1,000	FeSO ₄ · 7H ₂ O	40
DL-Aspartic acid	1,000	MnSO ₄ · 7H ₂ O	160
L-Arginine · HCl	200	NH ₄ Cl	3,000
L-Cystine	100	NaCl	40
L-Glutamic acid	1,000	Vitamins and growth factors	
Glycine	200	Adenine sulfate	10
L-Histidine · HCl · H ₂ O	100	Guanine · HCl	10
DL-Isoleucine	200	Uracil	10
L-Leucine	100	Xanthine	10
L-Lysine · HCl	200	Thiamine · HCl	1.0
DL-Phenylalanine	200	Pyridoxal	0.2
L-Proline	100	Ca pantothenate	1.0
DL-Serine	200	Riboflavin	1.0
DL-Threonine	200	Niacin	1.0
DL-Tryptophan	200	<i>p</i> -Aminobenzoic acid	0.2
L-Tyrosine	100	Biotin	0.01
Carbohydrate, buffer and salts		Folic acid	0.01
Glucose	20,000		
Sodium citrate	20,000		
Sodium acetate	1,000		
KH ₂ PO ₂	5,000		

Adjust to pH 6.8–7.0 with sodium hydroxide.

^a Henderson and Snell (131b). Possible effects of varying the composition of the medium are discussed by Snell (245); Schweigert and Snell (233) and Dunn (70b). See footnote 3.

of test tubes. To one series are added graded amounts of the amino acid to be assayed. This series will supply the standard reference curve (Fig. 1). To another series are added graded amounts of the solution containing unknown amounts of the amino acid to be assayed. The tubes are then sterilized and, after cooling, inoculated. After the proper period of incubation, the extent of bacterial growth is determined. In a satisfactory assay the apparent amino acid content of the unknowns will be the same at all levels on the usable part of the standard curve.

sources may contain cystine as an impurity (17). Samples of L-leucine have been shown to contain methionine (215). For these reasons it has been suggested that media be made up exclusively of synthetic amino acids. However, synthetic DL-leucine may contain DL-isoleucine as an impurity (131). Unknown contaminations may contribute to the uncertainties regarding the requirements of various organisms.

In some cases, it has been possible to supply the amino acid requirements of the organism in the form of a protein hydrolysate devoid of the amino acid to be assayed. Thus casein that has been hydrolyzed with acid is suitable for the assay of tryptophan (115), and glutamic acid-free (170), and methionine-free (181, 183, 215) casein hydrolysates have been found suitable for the assay of glutamic acid and methionine, respectively.

a. Measurement of response

Most of the constituents required for growth of an organism can be supplied; nevertheless, all of the materials which may *stimulate the rate* of growth are not yet known. These stimulating substances ordinarily do not affect the *amount* of final growth. Consequently, more consistent assay results are generally obtained

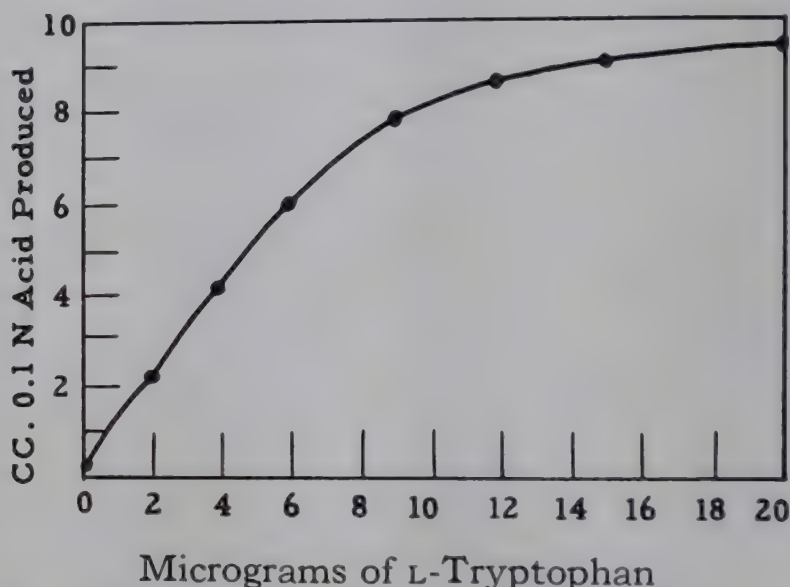


FIG. 1. Standard reference curve for microbiological assay (from Greene and Black, 115). The low blank titration in the absence of tryptophan and an extended straight portion of the curve are desirable features.

by the use of prolonged incubation periods. This necessity is responsible for one of the drawbacks of microbiological methods in routine analysis, namely, the length of time that must elapse between the setting up of the experiment and the determination of results. Recommended time periods are usually from two to five days. This disadvantage is partially overcome in a method described by Boyd, Logan and Tytell (32 a) who use a fast-growing organism, *Clostridium perfringens* BP6K. Incubation for 16 hours at 45° is a sufficient length of time for the assays.

With the lactic acid bacteria, two methods are interchangeably available for measuring the extent of growth of the organisms, namely, (1) titration of the lactic acid formed, and (2) photometric estimation of the turbidity of the medium (78, 125, 185). Titration can be made particularly sensitive by using a pH meter instead of indicators.

It has been suggested that it is more important to establish a few points accurately than to scatter the number of determinations

at different levels. In the "common-zero five-point assay" described by Wood (299, 89, cf. 78), single and double amounts of each preparation (standard and unknown) and a blank are used; the double amounts are at the highest levels that will still fall on the nearly linear part of the curve. In the ideal assay, the results can be plotted as two straight lines, and the ratio of their slopes is a measure of the relative content of the amino acid under assay.

The accuracy with which an amino acid may be determined in protein hydrolysates by microbiological methods is directly proportional to the percentage of the amino acid in the protein and, in general, inversely proportional to the amount of the amino acid required for the organism to produce a unit quantity of acid (cf. 219). In other words, accuracy increases with increasing amounts of amino acid relative to other amino acids added, and also with the sensitivity of the organism. Table IV indicates the levels of amino acids used in various assay methods with lactic acid bacteria.

In the microbiological assay of several amino acids, there is an initial lag in the dosage-response curve; the curve is sigmoidal in character. With glutamic acid, it has been assumed that this reflects a preliminary transformation of glutamic acid to glutamine before it can be utilized by the organism (see Glutamic acid). However, Shankman, Camien and Dunn (235) suggest that this and other "induction periods" may be due to specific antagonisms by amino acids closely related to those being assayed (alanine for glycine, tyrosine for phenylalanine, and threonine for serine). Meinke and Holland (193a) describe an antagonistic effect between serine and threonine. Each in large amounts inhibit the growth response due to the other.

TABLE IV
ASSAY RANGES FOR THE VARIOUS AMINO ACIDS^a

Amino Acid	Assay Range γ per 10 ml.	Amino Acid	Assay Range γ per 10 ml.
Alanine	40-500	Lysine	10-200
Arginine	10- 80	Methionine	10-100
Aspartic acid	50-500	Phenylalanine	10-150
Cystine	0- 50	Proline	10- 80
Glutamic acid	50-500	Serine	50-250
Glycine	10-100	Threonine	50-500
Histidine	5- 50	Tryptophan	2- 25
Isoleucine	10-100	Tyrosine	5- 80
Leucine	10-100	Valine	10-100

^a Snell (245, see also 78, 224). Micromodifications, in which the total volume required is less than 1 ml., are possible (245, 131c).

b. Limitations

In addition to the factors discussed in footnotes 2 and 3, the following limitations require mention:

As with other methods, when the microbiological methods are applied to protein hydrolysates, no measurement can be made of the amount of amino acid destroyed during hydrolysis. Complete hydrolysis is usually necessary since partially hydrolyzed materials may give results difficult of interpretation (170). In addition, since the micro-organisms usually respond only to the L-amino acids,⁴ racemization cannot be detected. For this reason, acid hydrolysis is usually preferred. For assays of tryptophan, proteins are hydrolyzed with enzymes or with alkalies (see Tryptophan).

Although Dunn and co-workers (78, 83) detected no changes in micro-organisms used for amino acid assay over a period of several years, this possibility has continually to be kept in mind. Cheldelin, Bennett and Kornberg (48) found that a strain of *L. fermenti*, used for the assay of thiamine, gradually developed ability to synthesize thiamine when it was grown on media containing only small amounts of this vitamin. When large amounts were furnished to the culture media, this tendency was repressed. Similarly, it may be necessary to supply carrying cultures with relatively large amounts of amino acids in order to suppress any synthetic potentialities in organisms to be used for amino acid assay.

The possibility that protein hydrolysates may contain not only measurable amounts of the amino acid to be assayed but also specific inhibitors has to be considered. Thus, Fox and co-workers (94, 90) found that D-leucine and D-valine, in amounts much higher than would ever be found in such media (100 times the

⁴ In establishing the validity of a microbiological method of assay, it is necessary to demonstrate that the response of the organism to the amino acid in question is specific. Usually a number of closely related compounds are assayed and found to have no activity. It is also desirable that other amino acids be tested individually in order to demonstrate that there is no response when only sub-optimal levels of the amino acid under assay are present.

In general, the D-amino acids have been reported to be entirely inactive, but D- and L-aspartic acid have been found to be equally effective for *L. delbrückii* (253) and the methionine isomers are interchangeable for *L. fermenti* (73). D-Glutamic acid has a small but definite growth-promoting effect for *L. arabinosus* on a medium deficient in glutamic acid (71, 170, 179, 125). Dunn, *et al.* (74) have determined the ability of several organisms to utilize D-glutamic acid in media deficient in L-glutamic acid. With two, there was no replacement; with seven, D- and L-glutamic acid were equally utilized; and with eleven, the response to the D-form was variable. The availability of these organisms of known response makes possible estimations of the D-glutamic acid content of protein hydrolysates by difference. Dunn, *et al.* found a casein hydrolysate to contain little if any D-glutamic acid, but the glutamic acid content of *L. arabinosus* cells was equally distributed between the D- and L-forms.

amount of the natural isomer present), acted as specific inhibitors for *L. arabinosus*. Waelsch *et al.* (287) observed that the sulfoxide and sulfone of methionine acted as specific inhibitors for the response of *L. arabinosus* to glutamic acid, and Ravel and Shive (211) showed that cysteic acid inhibits the growth of *L. casei*, *L. arabinosus*, and *L. mesenteroides*. Here the inhibiting agent is one that has been identified as a constituent of a protein material ("weathered wool" (61)). The inhibition by the sulfur compounds was overcome by aspartic and glutamic acids. Other derivatives and decomposition products in protein hydrolysates may exhibit similar types of inhibition (or stimulation), but have not yet been recognized. Dunn and co-workers (83) have noted that in a few cases *removal* of an amino acid from a complete medium resulted in an *increase* in acid production. Such observations attest to the importance of the proper balance of nutrients in the assay media (cf. 39a), and emphasize the point that, as with all biological assay methods, continual critical attention is essential.

c. *Neurospora* and *Tetrahymena geleii* methods

The fungus, *Neurospora crassa*, requires only sugar, salts, and biotin for growth. By irradiation, mutants have been prepared that require, in addition, single amino acids. For these strains, growth is a function of the concentration of the required amino acid in the medium, and the mold can be used for assay. This procedure has been applied to the assay for L-leucine (221a), L-lysine (70), and L-proline (23). Growth is usually estimated by weighing the mycelium formed during the incubation period of 7 to 8 days. A rapid method adapted to the assay of minute amounts (down to 0.01 γ)⁴ of amino acid is based on rate of germination (221).

Ryan and Brand (221b) noted that occasional flasks showed heavy growth of their "leucine-less" organism even in the absence of added leucine. This was considered to be due to spontaneous mutation to the wild form, and the results were discarded. If such mutations occurred during the latter part of an assay period, they might have escaped detection, and thus introduced an element of error into the results. No evidence of mutation was observed in the analysis of leucine by Hodson and Krueger (138), who obtained reasonable values even with intact proteins.

Rockland and Dunn (219) found that the tryptophan content of a protein could be assayed by permitting the ciliated protozoan, *Tetrahymena geleii* H, to grow in a medium containing a solution

of the intact protein. The organism requires tryptophan and elaborates sufficiently effective enzymes to make the tryptophan of the added protein available to it. This type of assay, if found dependable, would be even more useful than the others described, in that the destructive and racemizing effects of protein hydrolysis would be circumvented.

d. Criteria for reliability

Having established a standard curve, and then determined the amount of a particular amino acid present in the hydrolysate of a protein, one is faced with the difficulty of knowing whether to place reliance on the results so obtained. Snell (244) has suggested the following criteria: 1. There must be agreement in the assay results at different levels of assay, e.g., no "trends" should be apparent. 2. There must be agreement in the assay results when performed at different times, and if possible, under different assay conditions. 3. If a known amount of the amino acid being determined is added to the sample, it must be possible to account for it quantitatively. 4. If assay values obtained with two or more organisms are in agreement, the probability that the values are correct is substantially increased. 5. These values in turn should be in agreement with values obtained by entirely different methods of amino acid analysis.

3. Enzymatic Decarboxylation

In this method of amino acid determination, enzyme systems that have the property of specifically decarboxylating one amino acid are employed. An aliquot of a neutralized protein hydrolysate is mixed with such an enzyme system in a Warburg (98) or a Van Slyke apparatus (129) and the carbon dioxide evolved is measured by standard techniques. The total amount liberated is equivalent to the amount of amino acid present in the hydrolysate.

The decarboxylase enzymes that have been most thoroughly studied are those elaborated by bacteria. The information so far available has been summarized by Gale (100, 101). The enzymes are usually not natural constituents of the bacteria but are elaborated in the presence of the amino acid in response to an unnatural acidic environment. The formation of an amine and the liberation of carbon dioxide result in a net increase in pH to one more suitable for growth of the organism. For example, with *E. coli* grown on a simple medium containing only salt, glucose, and ammonia,

glutamic acid decarboxylase is the only amino acid decarboxylating enzyme present. If to this acidified medium is added 1% of one of the other amino acids for which the method is applicable, the corresponding decarboxylase is formed in significant amounts.

Gale and his co-workers (cf. 100) have studied the decarboxylase systems of more than a thousand strains of coli, streptococci, and other common organisms. From them they have chosen those that can be used for the isolation of specific enzyme preparations suitable for quantitative assay work (98, 99, 100). The strains used and some details concerning the preparation and properties of the enzymes are shown in Table V. After harvesting, the cells can be dried with acetone (except ornithine decarboxylase) and used in this crude form, or they can be further purified to yield preparations containing 50 to 100 times the activity of the dried cells. Extensive purification may result in the loss of activity due to separation of a coenzyme. This codecarboxylase has been identified as an orthophosphate ester of pyridoxal (121, 122). The bacterial histidine decarboxylase apparently does not require the coenzyme.

Highly active decarboxylases have been found thus far for arginine, lysine, histidine, glutamic acid, tyrosine, and ornithine. These amino acids are distinguished by having a polar group in the side chain. However, this is not the only requirement, since no active bacterial preparations have yet been found for aspartic acid or tryptophan (100).⁵

Each enzyme is specific for the decarboxylation of the natural isomer of the amino acid. All substituents so far investigated on the α -amino or carboxyl group or on the side-chain polar groups prevent decarboxylation. The substitution of an hydroxyl group for hydrogen in the substrate molecule other than on the polar groups, however, does not interfere with the reaction. Thus, hydroxylysine is slowly attacked by lysine decarboxylase, and synthetic beta-hydroxyglutamic acid is decarboxylated by the glutamic acid enzyme.

Schales and co-workers (225, 226, 227) have studied the glutamic acid decarboxylase content of higher plants, and found that extracts of carrots and squash are sufficiently active and specific to be used without further purification for quantitative determinations of L-glutamic acid. Codecarboxylase is required in this system (226).

⁵ However, the observations of Virtanen, *et al.* (284, 285a) indicate that an aspartic acid decarboxylase occurs in *Legume bacterium* (HX).

TABLE V
AMINO ACID DECARBOXYLASES (FROM GALE, 90A)

Enzyme	Organism (N.C.T.C. No.) ^a	Conditions of Culture		Specific Preparation	Activity Maintained	Manometric Estimation	
		Medium	Temperature (hr.)			Buffer	CO ₂ Evolution as % Theory
L-Lysine decarboxylase	<i>Bact. cadaveris</i> (6578)	3% casein digest 2% glucose (1 l. medium = 50 estimations)	25° 24	Acetone (5 vol.)-ether-dried powder, kept 3 days at 0° before use Use c. 10 mg. per test	4-6 weeks in desiccator	M 5 phosphate, pH 6.0 +acid-tip	92 98
L-Arginine decarboxylase	<i>Esch. coli</i> (7020)	3% casein digest 2% glucose (1 l. medium = 40 estimations)	25° 24	Acetone (5 vol.)-ether-dried powder Use c. 10 m.g per test	4-6 weeks in desiccator	M 5 phosphate- citrate, pH 5.2	95
L-Histidine decarboxylase	<i>Cl. welchii</i> , B.W. 21 (6785)	3% casein digest 2% glucose Heart muscle (1 l. medium = 20 estimations)	37° 16	Acetone (3 vol. for 30 min.)- ether-dried powder Use c. 30 mg. per test	2-3 months in desiccator	M 5 acetate, pH 4.5	96
L-Ornithine decarboxylase	<i>Cl. septicum</i> , PHII (547)	3% casein digest 2% glucose Heart muscle (1 l. medium = 20 estimations)	37° 16	Washed suspension of or- ganism 20-30 mg. dry weight cells/ml. Use 0.5 ml. per test	24 hr.	M 5 phosphate- citrate, pH 5.5 +acid-tip	92 98
L-Tyrosine decarboxylase	<i>S. faecalis</i> (6783)	3% casein digest 1% glucose 0.1% marmite (1 l. medium = 50 estimations)	37° 16	Acetone (5 vol.)-ether-dried powder Use c. 10 mg. per test	2-6 weeks in desiccator	M 5 phosphate- citrate, pH 5.5	96
L-Glutamic acid decarboxylase	<i>Cl. welchii</i> , S.R. 12 (6784)	3% casein digest 2% glucose Heart muscle Hydrogen (1 l. medium = 30 estimations)	37° 12	Washed suspension of or- ganism 20-30 mg. dry weight cells/ml. Use 0.5 ml. per test	48 hr. in H ₂	M 5 acetate, pH 4.5	98

^a National Culture Type Collection, London.

The enzyme decarboxylase methods seem to be rapid and capable of accuracy. Once the enzyme preparation is available, operation is extremely simple. So far, the method has been successfully applied, by workers other than Gale, only to lysine and glutamic acid (cf. 301, 227).

4. Chromatography

Chromatography has been defined as "a procedure of analysis by percolation of fluid through a body of comminuted or porous rigid material" (109). Originally developed as a method for separating organic solvent-soluble pigments, such as the chlorophylls and carotenoids, it has only recently been applied to the separation of water-soluble materials. The considerable number of papers on chromatographic methods for amino acids have been reviewed by Martin and Synge (192), Cannan (45), and Tiselius (266). Cannan (45) has discussed in detail the theory of chromatographic adsorption.

A system of partition chromatography was developed by Gordon, Martin and Synge (191, 104, 109) and was used by Tristram (269) for determining the monoamino, monocarboxylic acid contents of several proteins. The method is briefly as follows: The amino acids in a protein hydrolysate are acetylated with acetic anhydride. The resulting acetamino acids are then subjected to separations which involve partitions between an aqueous phase and a non-aqueous solvent mixture. The aqueous phase is held stationary in the form of a column of wet silica gel. A suitable indicator is adsorbed on the silica. The acetamino acids are then dissolved in the non-aqueous solvent and the solution is permitted to percolate down through the column. As it does so, the different amino acids move at different rates and so separate into bands that are detectable by means of color changes in the indicator. As the bands move out of the column, they are caught in separate containers and then subjected to further separation procedures, or to direct determination by titration (the acetamino acids can be titrated as acids).

In order to obtain reproducible and reliable data, it was necessary to pay careful attention to the preparation of the gel, to estimate the validity of the results by using control mixtures of amino acids simulating as closely as possible the mixture being analyzed, and to run a sufficient number of analyses to avoid fortuitous errors. Details were worked out particularly for the monoamino acids, alanine, valine, the leucines, phenylalanine, and proline. According to Tristram (269), it was not possible to separate leucine

from isoleucine by this procedure, but the sum of the two was determinable. The results for methionine were unsatisfactory. Partition chromatography has been used successfully to identify the amino acids of tyrocidine (108), gramicidin (108), and gramicidin-S (261). However, these substances contain only a few amino acids, compared to the usual protein.

Sanger (223) employed partition chromatographic methods to separate the dinitrophenyl amino acids obtained by hydrolyzing insulin, after it had been treated with dinitrofluorobenzene. The free amino groups of insulin were thus identified as belonging to glycine, phenylalanine and lysine.

The use of starch as the carrying column was suggested by Synge (260) and developed by Stein and Moore (250). In their procedures the amino acid solution is allowed to percolate slowly through the column, then collected in a number of fractions by an automatic fractionator. The most satisfactory solvent mixture contains butanol, benzyl alcohol and water. The amount of amino acid in each fraction is determined by a ninhydrin colorimetric method. When the resolving power is good enough to separate individual amino acids, a series of symmetrical distribution curves is obtained. The procedure has so far been applied only to phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine, and preliminary results indicate that it can also be used for the slower-moving amino acids. It appears that starch has several advantages over silica gel.

Partition chromatography has been used on a sub-micro scale by Consden, *et al.* (59). Cellulose in the form of a strip of filter paper is used as the stationary phase. The paper is suspended from a trough containing the solvent and the system is enclosed so that the atmosphere can be kept saturated with solvent. When a mixture of amino acids is placed on the paper near the trough, the amino acids migrate downward at different rates. Where two amino acids cannot be separated by one solvent, it is possible to turn the paper through an angle of 90° , and submit the partially separated amino acids to a second separation with a different solvent. By this means practically all of the amino acids can be separated from one another. At the end of a run, the paper is dried, sprayed with ninhydrin solution, and heated briefly. The amino acids show up as brown to yellow spots. This technique has been adapted to quantitative use (209a, 166a). It has already proven valuable in the demonstration that norleucine is *not* a normal constituent of pro-

teins (62). Five to 20 $\mu\text{g.}$ ⁶ of an amino acid gives a clearly visible spot. Dent (68a) has studied the behavior of some sixty amino acids and related compounds on phenol-collidine filter paper chromatograms (cf. 160a).

Bergdoll and Doty (24) have described an adsorption technique that appears to be accurate and convenient for separating the basic amino acids. The adsorbent is a mixture of Lloyd's reagent (Eli Lilly Co.) and a filter aid. The protein hydrolysate in dilute acid solution is first treated with zinc dust to reduce cystine to cysteine (cystine is adsorbed, cysteine is not), then washed through the acidified column. The basic amino acids are adsorbed. Lysine is eluted with hydrochloric acid, histidine with sodium bicarbonate, and arginine with pyridine. Each of the amino acids is determined by standard colorimetric methods. The method is simpler than that described by Wieland (243) in which several bleaching earths, Filtrol-Neutrol and Floridin XXF, are used.

The synthetic ion exchange resins are essentially solid acids or bases that therefore possess the capacity to hold bases or acids with which they come into contact. They are attractive analytical reagents because of the displacement phenomena by which a strong acid displaces a weak one or a strong base a weak base. A number of investigators have studied the possibilities of using such resins for the determination of amino acids (26, 44, 56, 61a, 87, 296) but the methods have not yet been developed sufficiently for routine use. As an example, a procedure proposed by Tiselius, *et al.* (267) includes adsorption of the aromatic amino acids on active charcoal, followed by adsorption of the basic amino acids in the filtrate on an ion-exchange resin containing carboxyl groups (Wolfatit C). The remaining amino acids are adsorbed on a resin containing sulfonic acid groups (Wolfatit KS), and after elution, the acidic amino acids are adsorbed on a resin containing amino groups (Amberlite IR-4).

The technique of following the separation of bands of colorless substances on columns rendered *fluorescent* either by the addition of unelutable dyes (40) or inorganic fluorescent materials such as zinc sulfide (234), has not yet been applied to amino acids.

The combination of chromatographic separation with other techniques may greatly increase the importance of this method. For example, if an amino acid mixture is reacted with radioactive "pipsyl" chloride, then separated on a paper chromatogram, the

⁶ The following are all used to indicate 0.001 mg.: 1 γ , 1 $\mu\text{g.}$, 1 microgram.

total radioactivity of each "spot" is a measure of the amount of amino acid corresponding to the spot. This type of work can be done with microgram quantities of proteins (158, 158a).

5. Ionophoresis

This term was proposed by Martin and Synge (192) to describe the movement of relatively small ions in an electric field. The term "electrophoresis" was reserved for the movement of large molecules and particles. Ionophoretic methods have been found useful for the quantitative separation, as groups, of the basic amino acids (arginine, histidine, lysine and hydroxylysine) and the acidic amino acids (glutamic and aspartic acids) from the neutral fraction of a protein hydrolysate. If a hydrolysate is placed in the middle compartment of a suitable three compartment cell, and a direct current is applied to electrodes placed in the end compartments, the basic amino acids pass through the membrane toward the cathode and the acidic amino acids pass through the membrane toward the anode. The efficiency of a cell is greatly dependent upon the membrane materials; vegetable parchment for the cathode membrane, and formalized gelatin on a cloth base for the anode membrane, have been used (189, 2). "Genuine" parchment is recommended (264).

Certain technical difficulties are encountered with ionophoresis which lessen its value as a method of amino acid separation. Among these are the following: a. Unless very low current densities are employed, considerable heat is developed, and cooling devices are required to secure an efficient rate of electrical migration. b. The migration of ions from the middle compartment tends to alter the pH of this compartment and, in turn, the ionic states of the amino acids. This may be counteracted by the continuous addition of buffering materials or by employing materials of different permeability as anode and cathode membranes so that cations and anions leave the middle compartment at equal rates. c. Amino acids tend to migrate from the middle compartment by diffusion as well as ionophoresis into the anode and cathode compartments. To secure good separations, therefore, the several fractions have to be reelectrolysed. d. One of the greatest difficulties attending the isolation of the acidic amino acids is that oxidation occurs at the anode with resulting serious losses.

A modification of ionophoresis was described by Sperber (247), who suggested that an ion exchange agent capable of adsorbing

acidic amino acids (Amberlite IR-4) be placed in the middle compartment of the ionophoretic cell together with the protein hydrolysate. At the end of the run, the basic amino acids are found in the cathode section, the neutral amino acids in solution in the central section, and the acid amino acids adsorbed on the ion exchange resin.

Consden, *et al.* (59, cf. 192) performed the ionophoresis in a slab of silica gel. The material to be analyzed was placed in a thin strip in the middle of the slab and current was applied at the two ends. The course of the separation could be followed by placing a piece of filter paper in contact with the slab, then "developing" it with a spray of ninhydrin solution. Separate bands showed up as red to purple colors. By careful attention to buffers and pH, it was possible to separate serine from glycine by this method.

6. Specific Precipitants

Most of the accumulated data on the amino acid composition of proteins were obtained by isolation methods (27). These depended upon precipitation by reagents, supposedly specific for single amino acids or groups of related amino acids. Further purification by recrystallization yielded fractions which, after having been characterized as pure by elemental analysis and melting point, could be weighed. Thus, information concerning the amounts of the amino acids present in the original protein was obtained. The efforts to correct for the residual solubilities of precipitates obtained with specific reagents generally were not successful, due to the influence on this solubility of the other amino acids, which varied in kind and amount for each protein. Stein and Moore (249) have evaluated precipitation techniques, and concluded that they cannot fulfill the requirements of primary standard methods. Their comment is apt: "In thus assessing these methods we should acknowledge our debt to them and to those who have worked with them. With these methods, information of incalculable value has already been obtained. In fact, the foundations of protein chemistry have been laid. It should occasion no surprise, however, that tools suitable for the laying of a foundation are not well suited to the fine architectural tasks entailed in completing the superstructure." By virtue of the extensive handling required, the precipitation methods are also not suitable for routine use. They will not be discussed in detail here (cf. 27, 192). The bulk isolation of amino acids for preparative purposes is discussed in Chapter IV.

Bergmann and his co-workers (196, 197) have developed an analytical system for amino acids which employed specific precipitants, but which was not a quantitative isolation procedure. This "solubility product" method was based upon the consideration that, in dilute solution, the solubility of salts of amino acids with specific precipitants (usually substituted aromatic sulfonic acids) conforms to the law of the solubility product; that is, the product of the concentration of the amino acid times that of the precipitant equals a constant. By determining the solubility of a sparingly-soluble amino acid salt in a solution of the protein hydrolysate, and again in a solution of the hydrolysate to which a known amount of the precipitant has been added, it should be possible to calculate the amount of the amino acid present in the hydrolysate. Unfortunately although the method has sound theoretical basis, it appears that technical difficulties limit its usefulness (249). Among these are (a) lack of availability of suitable reagents, (b) lack of constancy of the solubility product, and (c) requirement for expert manipulation. Slight experimental errors cause large deviations in the final result. In view of these disadvantages and the increasing availability of more convenient methods, it appears improbable that the solubility product method will find more than very limited use. However, the analyses that were made in the course of the development of the method appear to be in excellent agreement with those obtained by other reliable methods.

7. Miscellaneous

The methods discussed in this section use general group reagents for amino and carboxyl groups and are not specific for any individual amino acids.

a. Formol titration

Formaldehyde reacts with the amino groups of amino acids or peptides in neutral or alkaline solution to form methylol derivatives that are more acidic than the amino compounds themselves (Chapter I). The change in acidity is the basis of a method for titration of amino acids and protein split products. In a titration, the solution to be analyzed is adjusted to pH 6 to 7, 6 to 9% formaldehyde of the same pH is added, and the mixture is titrated with standard alkali to pH 9 (cf. 204, 275). This technique is useful for following

the hydrolysis accomplished by enzymes, acids or alkalies. With care, the reaction can be used to measure the purity of amino acids (77). However, since the ionization constants for formaldehyde compounds of amino acids, peptides, and proteins differ, it is not possible to interpret with any exactitude the meaning of the formol titration when it is applied to a mixture of protein hydrolytic products.

b. Amino nitrogen

Primary amino groups react with nitrous acid in acetic acid solution to yield nitrogen. The reaction forms the basis of Van Slyke's volumetric (270) and manometric (271) methods for measuring quantitatively the amino groups of amino acids and peptides. The manometric procedure is preferred, since it is capable of greater accuracy and requires less sample. The extent of hydrolysis of proteins may be judged by these techniques; amino nitrogen values are maximal when hydrolysis is complete. For greatest accuracy, ammonia, which reacts with nitrous acid to a variable extent (152), should be removed prior to the analysis (cf. 228). Proline and hydroxyproline do not yield nitrogen. Cystine and cysteine yield amounts in excess of the calculated quantities (cf. 192, 205). Glycine gives the calculated amount of nitrogen by the manometric procedure but excess amounts in the volumetric apparatus.

c. Ninhydrin reaction

The reaction of ninhydrin (triketohydrindenehydrate) with amino acids has been adapted for quantitative use by Van Slyke, *et al.* (272, 276, 277). Either ammonia or carbon dioxide can be determined, but the latter appears to give greater precision. The reaction is *specific for free amino acids*. Most peptides do not react. Glutathione is an exception since it contains a free amino group in the alpha position to a free carboxyl group.

Most amino acids yield exactly one mole of carbon dioxide and one mole of ammonia (cf. 272, 186, 246). Aspartic acid, however, yields two moles of carbon dioxide and one mole of ammonia. The reaction with asparagine is normal (200). Several investigators have used the difference between aspartic acid and glutamic acid (which reacts normally) to estimate the amounts of each present in fractions containing both compounds (272, 108, 45, 159).

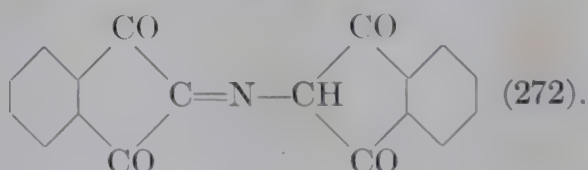
The use of hydrazine in the Van Slyke manometric procedure abolishes errors introduced by the volatile aldehydes from valine and the leucines (229, 127).

Determination of the ammonia evolved at pH 1–2.5 has been used by MacFadyen (186) and by Sobel (246). Proline and hydroxyproline do not yield ammonia, and tryptophan, cystine, and cysteine give anomalous results.

Virtanen, *et al.* (285, 286) investigated the possibility of using the volatile aldehydes resulting from ninhydrin oxidation of amino acids for quantitative purposes. It was found that alanine, leucine, isoleucine, valine, phenylalanine, and methionine form their corresponding aldehydes quantitatively (at pH 4–5), and that these can be separated by distillation and determined in the distillate by their bisulfite-binding capacity. The result gives an estimate of the total of the six amino acids. Acetaldehyde (hence, alanine) can be determined separately in the distillate by a colorimetric method (220, cf. 51).

The quantitative evolution of formaldehyde from glycine with ninhydrin at pH 1 has been suggested as a method for determining glycine (5, 187).

The color that forms above pH 2–3 when primary amino compounds are heated with ninhydrin is due to the reaction of the liberated ammonia with the reagent to form



Bergdoll and Doty (24) used the ninhydrin reaction colorimetrically to determine lysine after a chromatographic separation. The reaction with most amino acids can be made quantitative (196a).

Paper chromatograms of amino acids are developed by spraying with ninhydrin solution and then heating (59, 192) (see p. 76). It has been suggested that the spots can be developed faintly with dilute ninhydrin solution, cut out individually, eluted, and the color then determined quantitatively by the ninhydrin colorimetric technique (166a).

Moubasher (197a) has suggested that perinaphthindan, 2,3,4-trione hydrate be used instead of ninhydrin for the oxidation of amino acids, since it is cheaper and can easily be recovered.

III. APPLICATION TO SPECIFIC AMINO ACIDS

1. Basic Amino Acids

For many years methods for the determination of the basic amino acids followed the patterns originally set by Kossel, namely, precipitation of the group with phosphotungstic acid, followed by separation of the individual amino acids, for example, as their silver salts. The results so obtained are now known to be minimal values. The more recent methods often do not require preliminary separation of the basic amino acids. When this is desired, it can be efficiently and quantitatively accomplished by ionophoresis (electrodialysis) as described by Macpherson (189) and Theorell and Åkeson (264). Chromatographic procedures should be capable of similarly convenient separations (26, 24, 293). The phosphotungstate precipitation is known not to be quantitative (274), but can be used for the separation of the basic amino acids in isotope dilution procedures (238).

2. Arginine

A number of methods are available for the determination of arginine. The data are in remarkably good agreement, and it may be concluded that, of all amino acids, arginine is the most accurately determinable.

The very sensitive Sakaguchi color reaction is obtained when arginine in alkaline solution is treated with α -naphthol and sodium hypochlorite or hypobromite. In adapting this reaction for quantitative use, it has been found necessary to modify the technique in such a way that it is possible to develop maximum color, without concurrent destruction of arginine by excess hypochlorite (188, 4). Brand and Kassel (37) applied the reaction to the entire protein hydrolysate. Other guanidine compounds interfere, but these have not been shown to occur in proteins. Macpherson (189) estimated the arginine present in the catholyte fraction, obtained by ionophoresis of protein hydrolysates, by two methods: (a) his modification of the Sakaguchi reaction (188), and (b) determination of the ammonia liberated by boiling with alkali. The two methods gave closely agreeing results. The arginine content of a catholyte fraction has also been determined by precipitation as the flavianate (2).

The microbiological assays that have been described for arginine (see Table II) appear to be reliable and to give results in agreement with the other procedures. Preliminary announcement has been

made of the successful use of an arginine-requiring mutant of *E. coli* for arginine assay (245).

Much of the analytical data in the literature was obtained by preliminary separation of the basic amino acids followed by fractional separation of the individual amino acids (27, 192). Vickery (280) precipitated arginine as the diflavianate from the protein hydrolysate, then purified the precipitate by several recrystallizations as the monoflavianate. In this case, the amino acid derivative is insoluble enough so that apparently quantitative recoveries were possible, at least when arginine was present in amounts greater than 5% of the protein. Arginine was also isolated as the monoflavianate for its determination by the isotope dilution method (92).

The enzyme, arginase, has been used for the quantitative determination of the amount of arginine in protein hydrolysates. The urea formed can be estimated colorimetrically (8, 9, cf. 86), or by means of the carbon dioxide or ammonia liberated after the further action of urease (149, 151, 10). Other amino acids inhibit the rate of the reaction (150), but the excessive reaction time required with protein hydrolysates can be shortened by the use of manganese-activated preparations of arginase (9). The arginase methods are considered to be accurate and reliable, relatively simple, and moderately rapid (9).

A specific organism of the coliform group (Table V) furnishes arginine decarboxylase suitable for quantitative determinations of arginine (99). Reaction is complete in 20 to 30 minutes. Ninety-five percent of the theoretical amount of carbon dioxide is evolved; most of the remainder can be accounted for as being retained in the medium. Gale prefers to use a correction factor rather than to attempt to determine the residual carbon dioxide by the addition of acid. Agreeing data were obtained with entire protein hydrolysates and with the catholyte fractions obtained by Macpherson (189).

3. Histidine

Histidine reacts with diazotized sulfanilic acid in alkaline solution to give an intense red color (Pauly reaction). Of many adaptations to quantitative use, that of Macpherson (188, 180) appears most useful. Sulfanilamide gives 10% more color than does sulfanilic acid (56). Edlbacher *et al.* (85) determined histidine by coupling it with diazotized *p*-chloroaniline, then extracting the colored product with butyl alcohol. The color was stable and obeyed Beer's law. Unfortunately, tyrosine also gives a color with these

diazotized reagents, hence a preliminary separation is usually necessary. Macpherson (189) accomplished the separation by ionophoresis, but it has also been carried out by precipitating histidine as the phosphotungstate, or as the silver or mercury salt (27, 222).

Another colorimetric method used for histidine is based on the wine red color obtained when brominated histidine is heated (Knoop reaction). Kapeller-Adler (155) adapted the reaction for quantitative use, and it has since been modified by several investigators (cf. 27, 192).

The numerous papers on the separation of histidine by silver salt procedures have been summarized by Block and Bolling (27). The results for histidine are uniformly low compared to those obtained by the more recent methods. Vickery (281, 283) devised an isolation method based upon the specificity of 3,4-dichlorobenzenesulfonic acid as a histidine precipitant. Despite efforts to make this procedure capable of the accuracy necessary for standard data, the results of his analyses for proteins containing less than 5% histidine now appear to be 15 to 20% low (189, cf. 76). Macpherson (189) precipitated histidine from the catholyte fraction as the nitranilate (cf. 52). The amounts so accounted for were in agreement with the results obtained by the Pauly color reaction.

A number of microorganisms have been used to assay for histidine in protein hydrolysates (see Table II). In a painstaking study, Dunn and Rockland (78, 70a) concluded that high accuracy and precision were possible with *L. mesenteroides*. The histidine content of casein was found to be very close to 3.00%, and the value was considered to be sufficiently accurate to be adopted as the standard to which the other amino acid contents (of casein) could be referred.

The decarboxylase system for histidine is obtained from a strain of *Clostridium welchii* (Table V) (98).

4. Lysine

The isolation methods available for lysine, like those used with the other basic amino acids, gave results of doubtful significance. The amino acid was usually isolated as the picrate; the purity of this salt was judged by its explosion point. If the product exploded below 250° (pure lysine picrate explodes at 266°), recrystallization was advised (see 27, 52). For isotope dilution techniques, lysine has been purified as the ϵ -mono-, or di-benzoate after preliminary separations from other amino acids (92, 237, 238).

The decarboxylase method appears to be rapid and convenient. A specific strain of *B. cadaveris* (Table V) supplies the enzyme (98, 100). After 24 hours of growth, the cells are collected and dried with acetone. Ageing for a week or more allows a weak arginine decarboxylase activity to disappear. The Warburg (98, 301) or the Van Slyke manometric apparatus (129) may be used for the measurement of the evolved carbon dioxide. Gale (100) showed that hydroxylysine is also slowly decarboxylated by this enzyme, but recent evidence suggests that this amino acid is absent from most proteins (see Hydroxylysine).

Growth of the *Neurospora* mutant used for the determination of lysine (70) is inhibited by arginine, hence it is necessary to remove this latter amino acid, most satisfactorily by means of arginase, prior to the assay of protein hydrolysates. The data so far obtained by this method of assay are not in good agreement with the "best" values. Other microbiological methods are available (Table II).

Macpherson (189) separated the basic amino acids by ionophoresis, removed ammonia, and then estimated lysine from the difference between total nitrogen and that accounted for by arginine and histidine. Such procedures are usually to be distrusted since the results obtained may reflect errors in any of the other determinations. Nevertheless, Macpherson was able to account quantitatively for added lysine. Furthermore, estimations of lysine in the catholyte fractions by the decarboxylase method (98) were in excellent agreement with the results obtained by nitrogen difference.

Bergdoll and Doty (24) estimate the amount of lysine in a fraction obtained after chromatographic absorption, by development of color with ninhydrin (cf. 196a).

A lysine method based upon the blue color obtained when bromo- or chloro-lysine is added to phosphomolybdic-phosphotungstic acid (198) has not yet been adequately explored.

5. Hydroxylysine

The determination of hydroxylysine, as described by Van Slyke and coworkers (273), depended upon: (1) The precipitation of hydroxylysine with phosphotungstic acid, and (2) the quantitative estimation of the ammonia formed when this fraction was treated with periodate. Basic amino acid fractions of proteins, obtained by ionophoresis (189), were examined by Rees (212) for hydroxyamino acids by periodate oxidation (see Serine and Threonine). Of a number of proteins studied, only collagen and gelatin contained hydroxylysine (1.2 and 1.3%, respectively).

Hydroxylysine may be determined in the intact protein by estimating the amount of formaldehyde liberated by treatment with periodic acid (69). Serine at the amino end of a peptide chain would also yield formaldehyde under the same conditions.

6. Glutamic Acid

Isolation procedures have depended upon the insolubility of the barium or calcium salts of glutamic (and aspartic) acid in alcohol solution (Foreman procedure, see 27). In a critical study, Bailey, *et al.* (16) concluded that, as usually applied, this method gave results that were far from quantitative. By careful working up of residual fractions, this group of workers obtained data that they considered to be accurate to within 1 to 2% (49). It is of interest that the results so obtained were usually higher than those given by the isotope dilution or microbiological methods. Chibnall (49) attributes this fact to the specificity of the latter two methods for the L-isomer. The *isolated* amino acids were examined for optical activity and, by this criterion, were found to have from 0.1% (casein) to 9% (lactoglobulin) D-glutamic acid as percent of total glutamic acid. The significance of D-amino acids in protein hydrolysates has already been discussed (see p. 62).

For final purification, glutamic acid is crystallized as the hydrochloride from concentrated hydrochloric acid saturated with excess hydrogen chloride. If cystine is not removed in previous steps, it is only with difficulty removed from glutamic acid at this stage (237, 238).

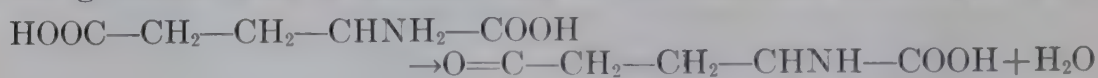
These isolation procedures have been used for the determination of glutamic acid by the isotope dilution method (277, 110, 237, 238).

In early attempts to use *L. arabinosus* for microbiological assay, it was noted that the standard reference curve was sigmoidal in character; that is, small amounts of glutamic acid appeared to have very little growth-promoting ability, and only after larger amounts were present, did the organism begin to respond to its presence (199, 170). The data suggested that glutamic acid is converted to glutamine before being utilized (179, 125). When small amounts of glutamine were included in the medium, a normal type response curve was obtained (179). However, acceptable assays are obtained with either type of assay medium. The addition of glutamine to the medium poses a separate problem, since this substance is readily converted to pyrrolidone carboxylic acid under the conditions used for sterilization. Guirard and Snell (118) propose, instead, to sub-

stitute small amounts of a tryptic digest of casein. This material satisfies the same requirements as does glutamine, but is stable to autoclaving. The inclusion of asparagine rather than aspartic acid in the medium also prevents the sigmoidal curves (19).

The decarboxylase methods appear to have many advantages. Gale (98) used a strain of *C. welchii* (Table V) as the source of enzyme, but the routine use of a pathogenic organism should be avoided if possible. A glutamic acid decarboxylase preparation from squash (227) is readily obtained, and gives analytical results in agreement with chemical and microbiological methods. However, a decarboxylase preparation from *E. coli* (269a) has the advantage of greater stability, and is more specific (157a).

When protein hydrolysates are autoclaved at pH 3.2, most of the glutamic acid is transformed to pyrrolidone carboxylic acid.



The resulting loss of amino nitrogen (corrected for cystine changes) has been made the basis for a quantitative method (205). Like all methods that depend on a small difference between two determinations of the same order of magnitude, it lacks precision, but it has been found useful for routine use.

Chromatographic procedures should be useful but have not yet been perfected. Cannan (45) adsorbed the dicarboxylic acids on Amberlite IR-4, then after elution, crystallized the glutamic acid as its hydrochloride. The results obtained were lower than the amounts of glutamic acid now considered to be present in the proteins used (cf. 159, 247). Consden, *et al.* (61a) separated glutamic from aspartic acid with a synthetic ion exchange column. Partition chromatography with silica gel was not satisfactory for the dicarboxylic amino acids, but pyrrolidone carboxylic acid was separable by this technique (128).

Glutamic acid can be estimated by conversion to succinic acid with chloramine T, followed by determination of the succinic acid manometrically with succinoxidase (57).

7. Aspartic Acid

The gravimetric methods used by many investigators for the quantitative estimation of aspartic acid in protein hydrolysates depended upon an initial precipitation of the dicarboxylic acids by the Foreman procedure (insolubility of the barium or calcium salts

in alcohol) followed by removal of glutamic acid, and finally the crystallization of aspartic acid as the copper salt (27). These methods were inadequate. The most painstaking analysis by such procedures indicated that β -lactoglobulin contained approximately 9% aspartic acid (52), whereas isotope dilution (92) and microbiological methods (124, 40) now indicate that 11% is closer to the true value.

The microbiological assay procedures are promising (see Table II). Stokes and co-workers (255) have shown that a close relationship exists between aspartic acid and biotin in connection with the growth of microorganisms. For many organisms, aspartic acid is required for growth only if the biotin content of the medium is limited. *L. mesenteroides* is an exception and is therefore the preferred organism for microbiological assay.

A specific deaminating enzyme, "aspartase," has recently been suggested for determining the aspartic acid content of protein hydrolysates (285a).

Chromatographic techniques for aspartic acid have been described, but reliable data for proteins have not yet been recorded (44, 45, 159, 294, 230, 68). The possible combination of ionophoresis and adsorption on an ion exchange reagent (247) has been mentioned (see Ionophoresis).

8. Amide Nitrogen

Most of the ammonia liberated during protein hydrolysis has its source in the amide groups of the glutamic and aspartic acid side-chains. These groups are more readily hydrolyzed than are peptide bonds, hence quantitative release of amide nitrogen requires less drastic conditions than does complete hydrolysis. Upon continued exposure to hydrolytic conditions, additional ammonia is liberated at a slow rate from decomposition of the other amino acids. Rees (212) has identified much of the non-amide ammonia nitrogen in protein hydrolysates as having its origin in the decomposition of serine and threonine.

Several techniques have been suggested for the determination of the true amide content of proteins. Shore, Wilson and Stueck (240) recommended hydrolysis with 20% hydrochloric acid for six to 10 hours at 100°, or three hours at boiling. Ammonia was then distilled from the hydrolysate made slightly alkaline with borate (pH 10) buffer (cf. 210). Rees (212), following the technique suggested by Gordon, Martin and Synge (104), maintained proteins in con-

centrated hydrochloric acid at 37° until the ammonia content of the hydrolysate was constant. Asparagine was 99% hydrolyzed in 220 hours, whereas in the same time, no more than 0.1% of serine nitrogen appeared as ammonia. Other investigators have used 2*N* hydrochloric acid at 100° for three hours (14, 177), or have autoclaved with 1.5*N* sulfuric acid at 120° for 40 minutes (96).

Ammonia is determined by distilling the hydrolysates made alkaline with borate buffer, or with magnesium or calcium oxides, preferably *in vacuo*. It is necessary to avoid strongly alkaline solution, high temperatures, or long-continued distillations in order to minimize opportunities for further release of ammonia from the decomposition of the remaining amino acids and peptides.

9. Tryptophan

Tryptophan is unstable toward oxidation by air under the conditions used both for acid and alkaline hydrolysis of proteins, consequently, special methods have been devised for its determination. The amino acid alone is comparatively stable to hot acids *in vacuo*, but is usually destroyed in acid hydrolysates of proteins due to oxidation and reaction with other amino acids and carbohydrates (207). Gramicidin, which contains 40% tryptophan and only a limited number of other amino acids (148, 260, 97), can be hydrolyzed by acid *in vacuo* without appreciable destruction of the tryptophan (54, 97, 146).

On the other hand, although alkaline hydrolysis has often been recommended for tryptophan determinations (35, 143, 135, 175, 130, 254), appreciable losses have been noted in some cases (117, 95, 164, 36, 300). Kuiken, Lyman and Hale (164) have identified this loss as due to destruction by air oxidation and find that it can be minimized by hydrolyzing the protein in the presence of added cysteine hydrochloride. Hydrolysis in an autoclave with 6*N* barium hydroxide for eight hours appeared to give optimal results (164). Enzyme hydrolysis has been used in several instances. Some techniques do not require preliminary hydrolysis.

Numerous workers have contributed to the elaboration of methods based upon the precipitation of tryptophan from hydrolysates as the mercury salt (27, 192). The precipitated complex is dissolved and its tryptophan content determined colorimetrically by the quantitative Millon reaction (174, 35), or by its ultra-violet absorption spectrum (33).

Most valuable of several colorimetric determinations is that based upon the reaction of tryptophan with *p*-dimethylaminobenz-

aldehyde and sodium nitrite in strong acid solution (113, 142, 236, 247b). This reaction can be used for the rapid and reliable determination of tryptophan in intact proteins (18, 256, 142, 243). In a detailed study with intact proteins, Spies (247a) showed that the rate of condensation of the reagent with the indole nuclei, the rate of oxidation of this complex with nitrite, and the quantity of nitrite required for maximum color development varied slightly with different proteins. He therefore recommended that optimal conditions should be separately determined for each protein. Such analyses were considered to have an accuracy of $\pm 1-3\%$, and a precision of $\pm 1\%$.

Other available colorimetric procedures are the following: Methods in which glyoxylic acid is the reagent give consistent results with hydrolysates and intact proteins (236, 256). Eckert (84) described a method based upon the reaction of tryptophan with nitrite and (1-naphthyl)ethylene diamine. Hauschild, *et al.* (130) compared this method with that of Shaw and McFarlane (236) (glyoxylic acid) and with a microbiological method. The last two gave reasonably agreeing results, but those obtained with Eckert's method were 10–25% lower. Recoveries of added tryptophan were quantitative from a casein hydrolysate, but only 80% from a gelatin hydrolysate.

A fluorometric method applicable both to intact and hydrolyzed proteins depends upon the reaction of tryptophan with 72% periodic acid (109a).

Tryptophan is readily determined by microbiological techniques (Table II). If alkaline hydrolysates are used, it is assumed that the tryptophan is completely racemized; consequently, only half of the original amount of L-tryptophan is present in the hydrolysate. Enzyme hydrolysis has been used with satisfactory results in several cases. The enzymes used have been pepsin and trypsin (300), papain (142), and pancreatin and hog mucosa (117, 164). The results obtained by Woolley and Sebrell (300) with *L. arabinosus* and *E. typhosus* were in excellent agreement with those obtained by the colorimetric method of Horn and Jones (with *p*-dimethylaminobenzaldehyde) (142).

The possibility of using *Tetrahymena geleii* for assaying the tryptophan content of intact proteins has already been discussed (see p. 71).

The amino acids responsible for the selective absorption by proteins in the ultra-violet region are tryptophan, tyrosine, and phenylalanine, but the last shows only weak absorption. By careful meas-

urement of the absorption spectra of proteins in 0.1*N* sodium hydroxide, Goodwin and Morton (103) were able to estimate their tyrosine and tryptophan contents.

10. Tyrosine

Both acid and alkali have been used to hydrolyze proteins for the determination of tyrosine. In the presence of excess carbohydrate, alkaline hydrolysis prevents loss of tyrosine through humin formation (120, 175). However, Hodson and Krueger (137) found a 10% loss in tyrosine even after barium hydroxide hydrolysis.

Tyrosine reacts with mercury salts and nitrite in acid solution to give a red color (Millon reaction). Colorimetric methods for tyrosine based upon this reaction have been used widely (91, 174, 175, cf. 27). The methods have been adapted for the spectrophotometer (35, 114). The chemistry of the reaction is complicated (174). Tryptophan interferes but can be removed as the insoluble mercury salt.

Thomas (265) adapted a color reaction between tyrosine and α -nitroso- β -naphthol for the quantitative estimation of the amino acid. The color is specific for para-substituted phenols. Since cystine appears to interfere, alkaline hydrolysis is preferable.

The Folin phenol reagent has been used to determine the combined tyrosine plus tryptophan contents of proteins after partial digestion by enzymes (95, cf. Chapter IX), and to determine tyrosine in acid hydrolysates. But in general, this reagent is not as specific for tyrosine, even in the absence of tryptophan, as the others already mentioned (206).

Gunness, *et al.* (120) described microbiological assays for tyrosine with *L. delbrueckii* and *S. faecalis*. The latter organism is inhibited at times by toxic substances occurring in alkaline hydrolysates of natural materials such as wheat. Both organisms give slightly higher tyrosine values when acid rather than alkaline hydrolysis is used, and also somewhat better agreement at different assay values. However, the values obtained are 10 to 15% below the values reported in the literature. It may be concluded that the absolute values for the tyrosine contents of proteins are still not known with certainty.

Tyrosine determinations so far reported by the specific decarboxylase technique are not as precise as those obtained with other amino acids by this method (98). A streptococcus organism is used (Table V). Its slight content of arginine decarboxylase is destroyed when the preparations are dried with acetone.

In the isotope dilution technique, tyrosine is crystallized from the protein hydrolysate following the addition of labeled DL-tyrosine. The L-amino acid is then separated as the copper salt (92, 237, 238).

A solubility product method was used to determine the tyrosine content of silk fibroin (251).

The spectrophotometric technique for determining the tyrosine and tryptophan contents of intact proteins has been mentioned (103) (see Tryptophan).

11. Phenylalanine

There is a comparatively wide divergence of values for phenylalanine in published analyses. Thus, Tristram (269) found 6.4% phenylalanine in *casein* by partition chromatography, but by microbiological assay, the values that have been obtained are: 5.3% (136), 5.0% (80), 5.9% (254), and 4.7% (17). By a new colorimetric method, Hess and Sullivan (134) found 5.8%, and a recent determination by the Kapeller-Adler method gave 4.8% (3). Such divergence of results is greater than would be expected from inhomogeneity of casein samples. These data appear to indicate that the result obtained by partition chromatography is high. Nevertheless, with *horse hemoglobin*, the value obtained by partition chromatography, 6.5% (269), is in fairly good agreement with the one isotope dilution value for phenylalanine so far available, 6.8% (92), and both of these values are considerably *lower* than the 7.5% obtained by microbiological assay with *L. arabinosus* (33).

At least part of the difficulty of assay may be ascribed to unknown and differing amounts of destruction of phenylalanine during hydrolysis. Block and co-workers (29) noted that somewhat more phenylalanine was detectable in alkaline than in acid hydrolysates. However, the apparently higher concentration of phenylalanine in the alkaline hydrolysate may have been due to its tryptophan content, since Knight and Stanley (162) found tryptophan to participate in the color reaction used. Bolling (cf. 27) observed that phenylalanine was destroyed by both acid and alkaline hydrolysis, although some protection was afforded in the presence of excess protein. Foster (92, cf. 238) found that 5.5% of phenylalanine was destroyed during 40 hours of boiling with 6*N* hydrochloric acid. Dunn, *et al.* (80) have stressed the possible critical role of conditions of hydrolysis in the determination of phenylalanine. Hess and Sullivan (135) suggest hydrolysis with 5*N* sodium hydroxide for two hours.

The Kapeller-Adler method (154), as modified by Block and Bolling (27), depends upon nitration of the phenylalanine with potassium nitrate in concentrated sulfuric acid, followed by development of color with hydroxylamine in ammoniacal solution (see also 3). Further modifications have been introduced by Grau (114). The recent Hess and Sullivan procedure (134) involves nitration, reduction, and coupling with naphthoquinone-4-sodium sulfonate. The ultimate usefulness of these methods remains to be determined.

Reference has been made to the microbiological methods (Table II). Hier, *et al.* (136) suggest that lack of agreement with chemical procedures indicates that some of the chemical values lack accuracy. However, the bioassays, also, appear to require development. Dunn and co-workers (80) note that recoveries, even from amino acid test mixtures, become less accurate with decreasing amounts of phenylalanine. They consider that their results with *L. mesenteroides* are more reliable than those with *L. casei* (used by Hier *et al.* (136)).

12. Leucine, Isoleucine, and Valine

Earlier methods for determining these three amino acids depended upon distillation of methyl esters (Fischer), or differential oxidation of the corresponding hydroxy acids (obtained by treatment with nitrous acid) to yield acetone (valine and isoleucine) and methyl ethyl ketone (leucine) (cf. 27). They proved to be entirely inadequate. Microbiological procedures appear now to be the most useful, particularly for routine analyses. The organisms that have been used are shown in Table II. The use of a leucineless *Neurospora* assay method has already been mentioned (221).

Leucine has also been determined by the isotope dilution technique. The leucine is isolated, after separation of the tyrosine from the protein hydrolysate, by direct crystallization from a concentrated solution, and purified, first by separation of its salt with 2-bromotoluene-5-sulfonic acid, and, finally, as the benzenesulfonyl derivative of leucine, prepared by benzenesulfonation of the leucine salt.

The valine and combined leucine plus isoleucine contents of several proteins have been determined by partition chromatography with silica gel (269) and the three amino acids separately with starch (250). The amounts of leucine and isoleucine present in fractions containing both can be determined by means of infra-red spectrophotometry (67a).

13. Proline

The several methods that have been used for the determination of proline include colorimetric and isolation techniques (see 27). The colorimetric methods are not reliable. Where analytically pure proline was isolated, at least minimal values were considered established. However, Stein (248) has pointed out the difficulties in accepting even this assumption.

The older isolation methods of analysis for proline depended upon its solubility in ethyl alcohol and butanol. Specific separations were accomplished by isolation of the copper salt, the methyl ester, the reineckate, or the rhodanilate. Approximations to their true proline contents were possibly obtained with proteins containing amounts over 10% (e.g., gliadin, zein, gelatin, casein), but with proteins having smaller contents, the results cannot be considered reliable.

Recently proline estimations in several proteins have been made by the partition chromatographic technique (269). Microbiological methods include the use of a "prolineless" *Neurospora* mutant and *L. mesenteroides* (23, 33). However according to the observations of Dunn, *et al.* (77a), *L. brevis* is the preferred organism.

14. Hydroxyproline

No satisfactory method for the estimation of hydroxyproline is known. Its absence from microbiological media results in no change of growth behavior with any of numerous organisms that have been tested, nor does its addition cause stimulation. Gelatin must contain appreciable amounts of hydroxyproline, for amounts in excess of 10% of the total weight have been repeatedly isolated from it, but the presence of hydroxyproline in proteins other than the few closely related to gelatin (collagen, isinglass) has not been proved. In this respect, hydroxyproline appears to resemble hydroxylysine, which occurs to the extent of approximately 1% in gelatin and collagen and is absent from many other proteins (212).

One proposed colorimetric method for hydroxyproline, based upon the oxidation and decarboxylation of hydroxyproline to pyrrole, followed by the development of a color with isatin or dimethylaminobenzaldehyde (cf. 27) is unreliable (214). A later modification (298b) may prove more useful. (See addendum).

15. Glycine

This simplest of the amino acids is one of the most difficult to determine analytically. Many of the data in the literature were

obtained by isolation techniques and are probably grossly in error except for those proteins that contain large amounts of glycine (20–50%) (Table VI). The isolation procedures depended upon the insolubility of the carbamate, the trioxalatochromiate, and the nitranilate (cf. 27). In some cases, other amino acids also are insoluble; in others, not all of the glycine precipitates.

Several colorimetric methods have been proposed. One procedure is based upon the green color that develops when glycine reacts with o-phthalaldialdehyde (209). The color is extracted with chloroform and measured. Tryptophan and ammonia interfere (cf. 27). A negative test was obtained with an hydrolysate of gramicidin (53), although glycine was later shown to be present (106).

The isotope dilution technique with pipsylechloride has been applied to glycine (see p. 64). This method will probably find use in the accumulation of standard data (158).

Glycine has been determined by the solubility product method (cf. 249), with results that appear to be reliable. The difficulties in the routine use of this method have already been outlined.

The assay of glycine with *L. mesenteroides* (235) does not have the precision of some of the other microbiological methods. For most purposes, however, it will be the most convenient technique to follow. MacFadyen (187) noted that glycine is the only amino acid to yield formaldehyde after reaction with ninhydrin. A quantitative method for the determination of glycine in blood and urine is based upon this reaction (5). Krueger (163a) found that it could be used with protein hydrolysates if interfering amino acids were first removed by adsorption on charcoal.

Glycine is a by-product of the acid decomposition of purines, hence the nucleic acid fraction should be removed from nucleoproteins before analysis for this amino acid (189a).

16. Alanine

Possibly the most accurate method for alanine is the pipsylechloride procedure (158) (see p. 64). Tristram (269) obtained alanine analyses by means of partition chromatography, and Sauberlich and Baumann (224a), by means of a microbiological method with *L. citrovorum*.

In the method of Virtanen and Rautanen (286) alanine is determined by the following procedure: The amino acids of a protein hydrolysate are oxidized by ninhydrin at about pH 4. The volatile aldehydes are distilled, and acetaldehyde is determined colori-

metrically in the distillate (220, cf. 298a). According to Alexander and Seligman (6), the oxidation should be carried out at pH 5.5 to avoid interference from aspartic acid.

17. Serine and Threonine

Both serine and threonine are slowly destroyed during the acid hydrolysis of proteins. Experiments with the isolated amino acids indicate that the amount of destruction (in boiling 20% hydrochloric acid, 24 hours) is equivalent to about 10% of the total serine and to about 5% of the total threonine (212, 32). However, destruction of these amino acids in peptide linkage may occur at a different rate. Serine in the form of its phosphoric acid ester undergoes considerable destruction during acid hydrolysis (67).

Satisfactory methods for determining serine and threonine depend upon oxidation with periodate (239, 201, 202). Protein hydrolysates are oxidized with periodate in alkaline solution. Acetaldehyde is aerated from the reaction mixture into a solution of bisulfite, then determined by standard procedures. Winnick (298) used a Conway diffusion cell for the transfer. Formaldehyde is not volatile if excess amino acids are present, but can be determined in the residual solution by precipitation with dimedon (201). Or it can be distilled from acid solution and determined in the distillate by bisulfite titration (212), colorimetrically with chromotropic acid (32, cf. 187), or by precipitation with dimedon.

In a careful study of these reactions as applied to protein analyses, Rees (212) has shown that the sum of the amino acids, determined by the aldehyde procedures, is generally in excellent agreement with the amount present as indicated by the amount of excess ammonia produced by the periodate oxidation (cf. 202, 273). Proteins that contain carbohydrate, such as egg albumin, yield formaldehyde somewhat in excess of the serine content. Under extreme conditions hydroxyproline also is attacked by periodate; formaldehyde, but no ammonia, is formed (46). Where carbohydrate is present, the best estimate for serine is based upon the difference between the total hydroxyamino acids (by ammonia) and the threonine (by acetaldehyde) (212).

Hydroxylysine, like serine, yields formaldehyde and ammonia when oxidized by periodate (273). In proteins containing hydroxylysine (gelatin, collagen), serine has been determined by the difference between the formaldehyde evolved by periodate oxidation of the unfractionated hydrolysate, and that accounted for in the basic amino acid fraction (212).

agreement with those obtained by a microbiological technique (*L. arabinosus* (144)). In the presence of excess cystine, there is rapid fading of the color (294).

Lavine's method (167) depends upon the reaction of iodine with methionine at pH 7 in the presence of excess iodide to give a compound that has not yet been clearly identified. Upon acidification, the compound reacts with iodide to give iodine quantitatively, which can then be titrated.

The microbiological methods that have been proposed are listed in Table II. The data of Lyman and coworkers (181) and those of Horn, *et al.* (144) are in good agreement with each other and with the colorimetric results. Unlike other organisms, *L. fermenti* utilizes D-methionine as readily as it does the naturally occurring isomer (73). Lampen and coworkers (166) use a mutant strain of *E. coli* (ATCC No. 9663) for the assay.

Several investigators (181, 215, 224) have taken advantage of the known destructive effect of hydrogen peroxide on methionine (268) to prepare methionine-free media for microbiological assay. Lyman, *et al.* (181, 183) treated peptone with hydrogen peroxide, then supplemented it with cystine, tryptophan, and tyrosine. Riesen and coworkers (215) used a hydrolysate of hydrogen peroxide-treated casein supplemented with cystine and tryptophan. For *L. mesenteroides*, additional proline was also necessary. No marked differences were noted from assays in which the medium contained pure amino acids instead of the treated protein or peptone hydrolysate. The advantage, particularly in cost, of hydrolyzed protein rather than purified amino acids in the medium is considerable.

Gordon, Martin and Synge (104) included methionine among the amino acids determinable by partition chromatography with silica gel, but Tristram (269) obtained erratic results in spite of repeated efforts to determine methionine by this method. Separation is satisfactory with starch columns (250).

Although methionine is usually determined in acid hydrolysates, Hess and Sullivan (135) recommended hydrolysis with 5*N* sodium hydroxide for 2 hours at 110°. The destruction of methionine in alkali, noted by Lyman and coworkers (181), is possibly minimized by the short hydrolysis period. Horn, *et al.* (144) reported excellent agreement in microbiological assay values, when proteins were hydrolyzed by acid and by an enzyme (papain), indicating that acid hydrolysis does not destroy methionine.

Lugg (177) and Evans (88) estimated the distribution of sulfur in proteins by oxidation with nitric acid, followed by the quantita-

tive determination of sulfate sulfur. Methionine sulfur was the difference between this sulfate value and the total sulfur, since methionine sulfur, unlike that of cystine and cysteine, is not converted to sulfate under such conditions (cf. 30, 43).

19. Cystine and Cysteine

The considerable literature on proposed methods for determining these two amino acids has been summarized elsewhere (27, 192). The unsatisfactory nature of many of the methods led Bailey (15) and Chibnall (50) to suggest that the best estimate of the cystine plus cysteine content of a protein may be obtained from the difference between the total sulfur and the methionine sulfur (see preceding section).

The difficulties arise at least in part from the labilities of cystine and cysteine under the conditions of hydrolysis. Both are destroyed by alkaline hydrolysis; cysteine is also unstable in acid. The instability of cysteine is enhanced in the presence of carbohydrate (173). Halwer and Nutting (126) found that added cysteine could not be quantitatively recovered after acid hydrolysis of β -lactoglobulin, or even after being held at room temperature with an acid hydrolysate, although heating in the presence of a mixture of amino acids simulating the protein hydrolysate did not cause destruction. Hydrolysis in an atmosphere of carbon dioxide or with urea did not improve the recoveries. These data indicate that all reported analyses of cysteine after acid hydrolysis should be suspect. In addition to this difficulty, it has been shown (207) that cysteine is *formed* from cystine during hydrochloric acid hydrolysis of proteins rich in tryptophan. It is thus clear that, during acid hydrolysis of most proteins, there is both formation and destruction of cysteine, and that the amounts so formed and destroyed probably vary from one protein to the other. Hess and Sullivan (132) have reported a series of analyses in which they indicated that the cysteine contents of hydrolysates were in good agreement with the SH contents of the original proteins as measured by iodine titration in dilute acid, but these data may have been the result of compensating errors.

Of the colorimetric methods that have been devised for determining cysteine or cystine plus cysteine in protein hydrolysates, the following have been found by several investigators to give values reasonably in agreement. In general, the color is developed with cysteine. To obtain cystine plus cysteine values, the cystine

is reduced prior to the development of the color. When zinc (278, 193) or sodium amalgam (257) are used for the reduction, the cystine is entirely reduced to cysteine, but if cyanide or bisulfite is used, the reaction is more complicated since these reagents add to, rather than directly reduce, the disulfide bond.

a. Folin reaction

Cysteine reduces phosphotungstic acid (Folin's uric acid reagent) to give a blue color. Various modifications (156, 13, 172) of this method have been used to study the sulfur distribution in several purified proteins (cf. 33, 241). Agreement between the total sulfur as determined analytically and as calculated from the combined methionine, cystine, and cysteine contents is usually good.

b. Sullivan reaction

Sullivan determined cysteine by its very specific color reaction with 1, 2-naphthoquinone-4-sodium sulfonate in a strongly reducing medium. Cystine and cysteine are distinguished (257) by comparing the color obtained after complete reduction (with sodium amalgam) to that obtained after reaction with cyanide, in which case only one half of the cystine is determinable. Csonka, *et al.* (65) found that prior precipitation of the cystine and cysteine with cuprous oxide (cf. 21) led to more consistent results by the Sullivan method. Altogether, the numerous modifications that have been proposed indicate that this technique has not yet been developed to an entirely satisfactory state.

c. Vassel method

Cystine reacts with *p*-aminodimethylaniline in the presence of iron and zinc to give a red color. The method is capable of ready duplicability (278, 193) but has not been widely used.

d. Iodometric method

The oxidation of cysteine by iodine has been used for the determination of cystine in proteins (cf. 27). The cystine in a hydrolysate is reduced to cysteine with zinc, and then titrated with standard iodate-iodide. Results may vary widely, depending upon the exact conditions used (cf. 171).

Nakamura and Binkley (197b) found that a blue color was obtained when cysteine was treated with brucine, 50% sulfuric acid

and potassium persulfate. The reaction is said to be more specific than the Sullivan reaction, and to be applicable to protein hydrolysates prepared with sulfuric acid.

Cystine has been determined microbiologically with *L. arabinosus* (17) and *L. mesenteroides* (131a, 224). In some cases, difficulties have arisen due to the presence of cystine as an impurity in the amino acids used in the basal medium. Barton-Wright (17) recommended the use of synthetic amino acids wherever possible. Sauberlich and Baumann (224) obtained consistent results (in assays of urine) only when the bulk of the amino acids in the medium was supplied by a hydrogen peroxide-treated casein hydrolysate (268). It appears that microbiological assays for cystine in protein hydrolysates may be of doubtful value in view of the rapid racemization of cystine by acids (139, 140), and its destruction by alkalies (cf. 215a).

Cystine and cysteine are precipitated when heated in solution with a suspension of cuprous oxide. The cuprous oxide reduces the cystine to cysteine, which then forms the insoluble cuprous mercaptide. Several investigators have used the procedure for the quantitative determination of these amino acids (112, 231, cf. 192), and also to remove cysteine and cystine from solutions prior to the determination of other amino acids (16, 92, 237, 238). The precipitate may contain other amino acids (cf. 16), or purines (from nucleoproteins) (cf. 231).

The difficulty in interpreting the cysteine content of hydrolysates has already been discussed. It appears that the most suitable methods for determining the cystine and cysteine contents of proteins may be as follows: Cysteine should be determined on the intact, or possibly the enzyme-digested (7) protein (see Chapter IX). Total cystine plus cysteine should be determined in a hydrolysate so treated that amounts of cysteine present at any one time are minimal. Mirsky and Anson (195) proposed to oxidize the SH groups of proteins with hydrogen peroxide before hydrolysis in order to determine cystine in the hydrolysate. However, Lugg (173) was unable to find an oxidizing agent suitable for this purpose.

In their study of sulfur distribution in proteins, Hess and Sullivan (132) could account for only 82 to 87% in three proteins (cf. 241). However, it was shown that the entire sulfur content of the hydrolysate was in the form of methionine, cystine, or cysteine; hence, it was not necessary to assume the presence of an unidentified sulfur amino acid. The loss of sulfur was thought to occur during hydrolysis, possibly as a volatile mercaptan.

20. Miscellaneous

Lugg (175) and Brand and Kassell (35) measured diiodotyrosine and thyroxine in proteins by determining the increase in tyrosine color after alkaline-stannite, as compared to alkaline hydrolysis. Only the former system accomplishes reduction of diiodotyrosine and thyroxine to chromogenic substances. The amount of color produced by each differs, hence their relative amounts can be estimated by solving two simultaneous equations in which total iodine and increase in Folin values are the determinable factors.

A more suitable method for thyroxine is based upon its selective extraction with butyl alcohol from an alkaline hydrolysate, followed by determination of iodine in the extract (cf. 176). Diiodotyrosine is not extracted by butyl alcohol under these conditions. Roche and Michel (218) used the color obtained with sodium nitrite to determine diiodotyrosine and thyroxine after the separation with butyl alcohol.

Ornithine has been identified by chromatographic separation (108, 217). A specific decarboxylase preparation is available (Table V) for this amino acid (99). Ornithine is a natural constituent of tyrocidine (108) and gramicidin-S (217), but has not yet been identified in proteins.

Many purified proteins contain constituents other than amino acids. Discussion of methods for their determination is outside of the scope of this chapter. In general, carbohydrates are determined by reaction with orcinol or carbazole in strong sulfuric acid (215, 123, 141), hexosamines by their reaction with acetylacetone (208), and lipids by extraction with alcohol or acetone (47).

IV. AMINO ACID COMPOSITION OF PROTEINS

1. Method of Recording Results

The simplest method of expressing the results of amino acid analysis, and that most generally used, is in terms of "per cent" or "grams amino acid per 100 grams of dry and ash-free protein." These data are partly misleading, since amino acids do not occur as such in proteins, but instead, lack the elements of water. Thus, all of the amino acids in a protein would account for 110 to 120%, rather than 100%, of the weight of the protein. The total would depend upon the average molecular weight of the amino acids present, and this would not be exactly known until the analysis was complete.

In order to circumvent this difficulty, results may be expressed

as "grams of amino acid residue per 100 grams protein," the residue weight being equal to the molecular weight of the amino acid minus 18, the molecular weight of water. In this case, if the amino acids of a protein were completely known, the sum of the data, so expressed, should equal 100 grams.

Martin and Synge (192) and Chibnall (50) have urged that investigators use "per cent amino acid nitrogen of the total nitrogen" or "grams amino acid nitrogen per 100 grams nitrogen." The advantages are the following: As in the method mentioned above, the sum of the amino acid analyses becomes equal to 100%. Furthermore, expressed in this fashion, the data are independent of the nonnitrogenous constituents of the protein preparation used, such as, for example, its moisture, ash, or carbohydrate contents. Comparisons of similar protein preparations of differing nitrogen contents are thus facilitated. Given the nitrogen content of the preparation, it is a simple matter to recalculate data to any of the other methods of expressing results.

The ideal mode of expression would be in terms of "moles amino acid per mole protein"—These would be simple whole, but sometimes very large, numbers. Since there is little agreement as yet concerning the true molecular weights of the various proteins, recourse is had to expressing the results for some arbitrarily chosen mass of protein. The results then appear as "gram-molecules amino acid per 10^4 (or 10^5) grams of protein." Data presented in this manner permit direct addition of groups. For example, the total gram-moles of basic groups per 10^4 grams protein would be the sum of the number of gram-moles of lysine, arginine, histidine, and free alpha amino groups per 10^4 grams protein (see Chapter IX). "Millimoles amino acid per gram protein" and "gram-moles $\times 10^3$ per 100 grams protein" are also used.

In amino acid analysis of feeds and foods, it is sometimes convenient to express results in terms of "grams of amino acid per 16 gm. nitrogen (roughly per 100 gm. protein)" in order to permit direct comparison of the amino acid compositions of various nitrogenous materials (27, 28). Such practice should not be followed with purified proteins (282), since their nitrogen contents may range from 10 to 20 per cent.

2. Amino Acid Content of Proteins

In Table VI are listed the amino acid compositions of several proteins that have been analyzed in some detail. Only the more

TABLE VI
PERCENTAGE AMINO ACID COMPOSITION OF PROTEINS^a

	Molec- ular Weight	Nitro- gen Content	Casein ^b	Edestin	Egg Albu- min	Gela- tin ^b	Gliadin	Horse Hemo- globin	Insu- lin	β -Lacto- globulin	Myosin	Serum Albumin (Bovine)	Silk Fibroin	Tobacco Mosaic Virus	Type A Toxin (Cl. botu- linum)	Wool	Zein
Total Nitrogen																	
Alanine	89.1	15.72	15.7	18.7	15.8	18.1	17.7	16.8	15.8	15.6	16.8	16.1	18.7	16.6	16.3	16.3	16.2
Ammonia	17.0	82.35	3.0	4.3	6.7	9.3	2.1	7.4	4.7	7.4		5.0	31	5.1	3.9	4	10.5
Arginine	174.2	32.16	1.7	2.2	1.3	0.1	5.5	0.9	1.9	1.3	1.5	1.0	0.1	1.6	2.6	1.4	3.6
Aspartic acid	133.1	10.52	4.1	17.4	5.7	8.6	2.6	3.7	3.2	2.9	7.4	6.2	1.1	9.8	4.6	10.2	1.7
Cysteine	121.1	11.57	7.2	13.4	9.3	6.8	3.3	10.4	7.5	11.3	8.9	11.1	2.8	13.5	20.3	7.3	4.6
Glutamic acid	240.3	11.66	0.4	0.5	1.3	0	0	0.5	0	0.8	1.2	0.3	0	0.7	0.2	0	0.8
Glycine	147.1	9.52	22.4	19.4	0.6	0.1	2.6	0.5	12.4	2.6	0.2	6.2	0.2	0	0.6	11	26
Histidine	75.1	18.66	2.7		16.0	11.0	46.0	8.5	18.4	21	22	16.9	2.0	11.3	15.6	15.3	
Hydroxyproline	155.2	27.08	3.0	2.6	3.1	25.5	2.1	5.6	4.4	1.5	1.9	2.0	44	1.9	1.4	6.5	
Hydroxylysine	131.1	10.68	0	0	2.4	0.7		8.4	5.0	1.6	2.4	3.8	0.4	0	1.0	1.1	1.3
Isoleucine	162.2	17.26	0	0	0	1.3	0	0	0	0	0	0	0	0	0	0	0
Leucine	131.2	10.68	6.1	4.7	8.0	1.7	5	0	2.9	6.1	0	2.6	0	6.6	11.9	0	6.9
Lysine	146.2	19.16	9.2	7.4	9.6	3.5	6.5	15.1	13.4	15.7	15.6	12.3	0.8	9.3	10.3	11.3	15.4
Methionine	149.2	9.39	8.2	2.3	6.1	4.6	1.1	8.6	2.5	11.4	12.0	12.4	0.7	1.5	7.7	2.7	0
Phenylalanine	165.2	8.48	3.0	2.4	5.0	1.1	1.7	1.5	0	3.2	3.4	0.8	0.15	0	1.1	0.7	1.5
Proline	115.1	12.17	5.5	5.5	7.4	2.3	5.7	6.8	8.0	3.8	4.3	6.6	1.3	8.4	1.2	6.7	7.0
Serine	105.1	13.33	11.6	4.6	4.1	14.2	13.4	3.9	2.8	5.4	1.9	5.6	0.7	5.8	4.4	10.7	10
Threonine	189.1	11.76	5.9	6.3	8.1	3.2	4.9	5.8	5.5	4.1	4.4	4.5	16.2	7.2	4	10.0	7.0
Tryptophan	204.2	13.72	4.5	3.9	4.1	2.2	2.1	4.4	2.1	5.1	5.2	6.5	1.6	9.9	8.5	6.4	3.4
Tyrosine	181.2	7.73	1.7	1.6	1.4	0	0.6	1.2	0	2.6	0.8	0.6	0.4	2.2	1.9	1.5	0.1
Valine	117.1	11.96	6.1	4.3	4.0	0.5	3.1	3.1	13.3	3.8	3.2	5.2	12.0	3.8	13.5	4.8	5
Amino acid nitrogen as per cent of total nitrogen			99	95	98	92	93	96	98	98	85	102	97	100	100	95	89

^a On a moisture and ash-free basis.
Most of the figures in this Table have been taken from the following references: 25, 33, 39, 41, 50, 92, 98, 99, 161, 189, 190, 197, 199, 212, 237, 238, 241, 242, 250, 269, and from papers referred to in Table II. Probably the greatest accuracy should be assigned to the arginine values (50, 189). Values for histidine, lysine, methionine, serine, threonine, tryptophan, and tyrosine are possibly correct within $\pm 10\%$, the others to $\pm 20\%$. See Tristram (268a) for discussion of recent amino acid analytical data.
These data may be expressed in other ways as follows: To change "percent" to "number of gram moles per 10³ gm. (or 10³ gm.) of protein," divide the figure in the Table by the molecular weight of the amino acid (Column 1) and multiply by 100 (or 1000). To obtain "amino acid nitrogen as percent of total nitrogen," multiply the figure in the Table by the nitrogen content of the amino acid (Column 2), and divide by the nitrogen content of the protein (Line 1).
^b The amino acid composition of gelatin will vary with its source and method of preparation (cf. 27, 50). Casein, also, has been criticized as not possessing the constancy of composition preferred in a standard reference protein (cf. 50, 78).

recent and, it is to be hoped, reliable data have been included. For discussions of the interpretation of such analytical findings, see 49, 50, 58, 33. A somewhat different picture of the protein molecule is given when the results are expressed in terms of polar groups, as given in Table I, Chapter IX.

Addendum

More recent developments in the analysis for a number of amino acids are mentioned below.

Hydroxyproline is determined by oxidation with alkaline hydrogen peroxide and cupric ion, followed by acidification and reaction with *p*-dimethylamino benzaldehyde. An intense red color develops. (Neuman, R. E., and Logan, M. A.: *J. Biol. Chem.*, 184: 299, 1950.)

A new procedure for estimation of aspartic acid depends upon conversion of aspartate to fumarate with dimethyl sulfate. Fumarate is precipitated as the copper-pyridyl complex. The copper in the complex is then determined by a colorimetric procedure. (Friedburg, F., and Marshall, L. M.: *Proc. Soc. Exper. Biol. Med.*, 74: 446, 1950.)

Data obtained by chemical (64) and microbiological methods for determining methionine have been shown to give agreeing results with meat hydrolysates. (Schweigert, B. S., Guthneck, B. T., Kraybill, H. R., and Greenwood, D. A.: *J. Biol. Chem.*, 180: 1077, 1949.)

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Chapter IIIA

THE PREPARATION OF AMINO ACIDS AND POLYPEPTIDES

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A. AMINO ACIDS

ALMOST one hundred years ago the first synthesis of amino acids were reported by Strecker and Dessaignes. Since that time a number of general methods have been devised and perfected by a large number of chemists. Fischer, Sørensen, Erlenmeyer, Leuchs, von Braun, Harington, Robson, Abderhalden, Marvel, Sasaki, and many others have made notable contributions in the field of preparative amino acid chemistry.

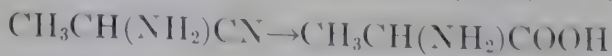
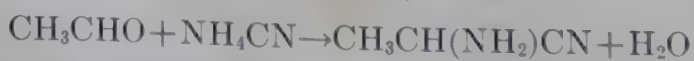
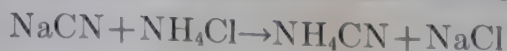
After a brief period of inactivity, developments in the fields of nutrition and chemotherapy resulted in a resurgence of interest in the preparation of amino acids and within the past few years several noteworthy advances have been made. Thus, with the possible exception of DL-arginine, DL-hydroxyproline, and DL-thyroxine satisfactory syntheses for all the amino acids of biochemical interest are available. The preparation of individual amino acids is discussed after a brief treatment of the general synthetic methods. Although limitations of space preclude the possibility of summarizing all the pertinent literature it is hoped that the best preparative methods are adequately covered.

I. General Methods for the Preparation of Amino Acids

1. Strecker reaction

In 1850 Strecker prepared DL-alanine by treating aldehyde-ammonia with hydrogen cyanide and then hydrolyzing the resulting aminonitrile to the amino acid (251). This method has been critically examined by Cocker and Lapworth (53) who recommended that alkali metals be avoided and hydrogen cyanide be used to insure the best yields of the more soluble amino acids. They succeeded in preparing DL-alanine in 75% of the theoretical yield.

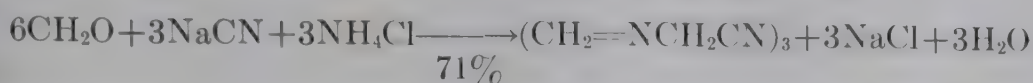
To avoid handling hydrocyanic acid a technique has been devised which generated the required ammonium cyanide *in situ*. Kendall and MacKenzie (195) prepared DL-alanine from acetaldehyde, sodium cyanide and ammonium chloride.



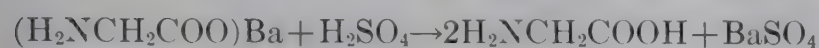
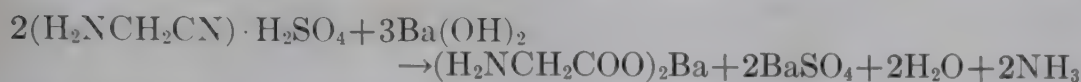
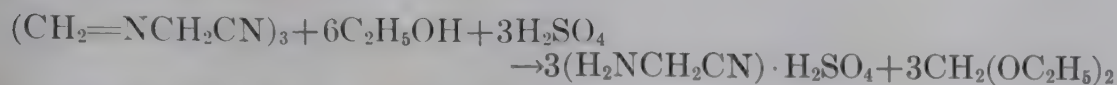
They were able to obtain the amino acid in 60% of the theoretical yield.

When formaldehyde was treated with ammonium chloride and

sodium cyanide the trimer of methylene-aminoacetonitrile separated.



Glycine (196, 199) was obtained in two steps and in 70% yield based on the trimer.

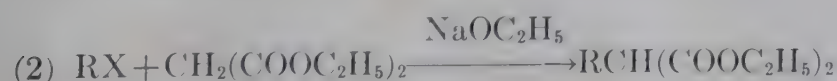
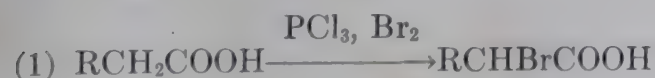


The Strecker synthesis has been applied also to the preparation of DL-leucine (72), DL-methionine (18), DL-norleucine (153), DL-phenylalanine (94), DL-valine (178), DL-serine (116), and DL-glutamic acid (67, 161).

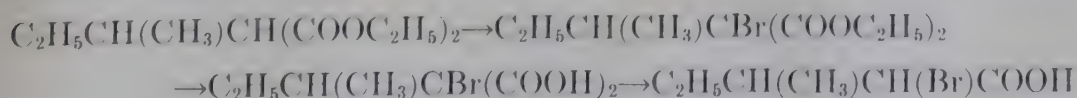
A severe limitation to the generality of this amino acid synthesis is the inaccessibility of the required aldehydes. The inherent danger in working with large quantities of cyanide and the tedious isolation procedures often encountered tend to make other methods of preparation more attractive.

2. Amination of halogen acids

In 1858, Cahours (38) and Perkin (211) prepared glycine by the amination of chloroacetic and bromoacetic acid, respectively. Since that time this method was recognized as being of considerable utility and has been applied to the synthesis of a wide variety of amino acids. The required halogenated acids are usually obtained by either of two routes.



A slight variation in this method was used by Romburgh (223) in his synthesis of DL-isoleucine. The malonic ester was brominated before saponification.



It was not until long after the amination method was in general use that careful studies of the reaction were undertaken. Robertson (222) believed that the concentration of chloroacetic acid should not exceed one mole per cent of the ammonia concentration in order to prevent side reactions. Chadwick and Paes (49) determined the rates, activation energies and entropies of the reaction of chloroacetic, bromoacetic, bromopropionic and *n*-bromoiso-caproic acids with ammonia. Conditions for the realization of optimum yields could be determined from their data.

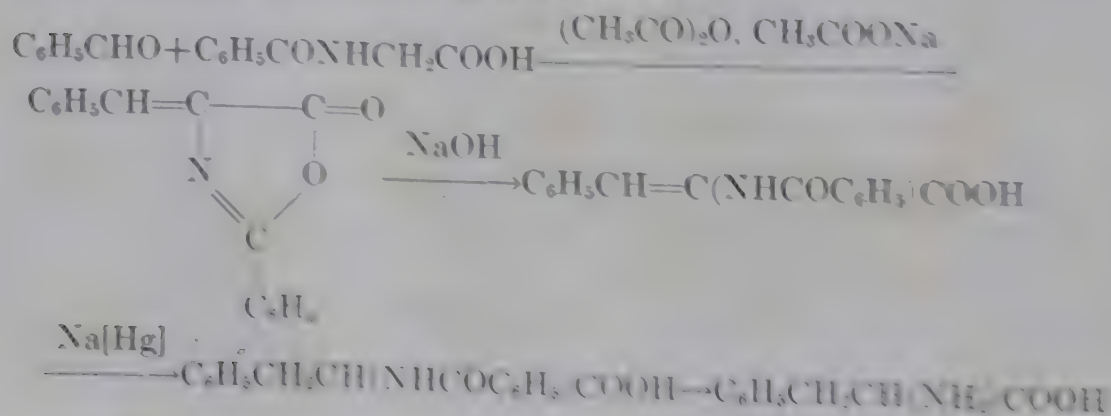
Cheronis and Spitzmoeller (50) and Dunn, Butler and Frieden (65) studied the effect of ammonium carbonate on the amination reaction. The amount of ammonia needed could be diminished considerably, and higher yields were obtained when ammonium carbonate was added to the reaction mixtures. These authors suggested that a complex, such as $\text{HCO}_2\text{CH}_2\text{NHCO}_2\text{NH}_4$, was formed which prevented further reaction from taking place.

Excellent preparative methods for glycine and DL-alanine have been reported by Orten and Hill (206) who obtained the former amino acid in 64% yield from chloroacetic acid. Tobie and Ayres (255) improved the method and increased the yield to 77%.

The experience of various workers has led to the conclusion that at higher temperatures shorter reaction times may be used, that a large excess of ammonia minimizes by-product formation, and that ammonium carbonate exerts a beneficial influence on the course of the reaction.

3. Syntheses from aldehydes

Plöchl (213) showed that benzaldehyde condensed with hippuric acid to give a substance that upon reduction and hydrolysis yielded DL-phenylalanine. However, it remained for Erlbaumeyer (80, 90) to clarify the steps in the synthesis and point out the usefulness of the method. The reactions involved are:

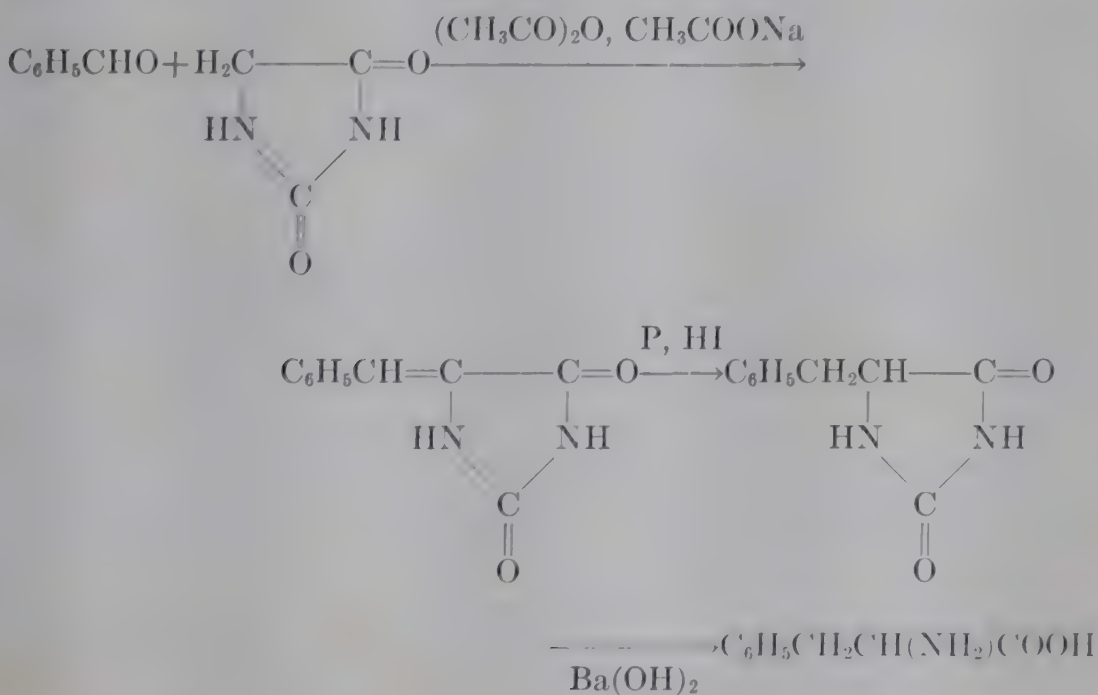


Simultaneous reduction and hydrolysis of the azlactone may be carried out with phosphorus and hydrogen iodide in acetic acid solution (142, 169). DL-Phenylalanine has been prepared in 42% overall yield with this modification (201).

Erlenmeyer (82) showed that aceturic acid condensed with benzaldehyde to give the corresponding azlactone and Herbst and Shemin (202) completed the synthesis. α -Acetamidocinnamic acid was reduced catalytically and the product deacetylated to give DL-phenylalanine in 54% overall yield.

Best results in the azlactone synthesis have been realized with aromatic aldehydes although isobutyraldehyde and acetaldehyde have been used (93, 47).

Wheeler and Hoffman (267) used hydantoin to prepare aromatic amino acids.

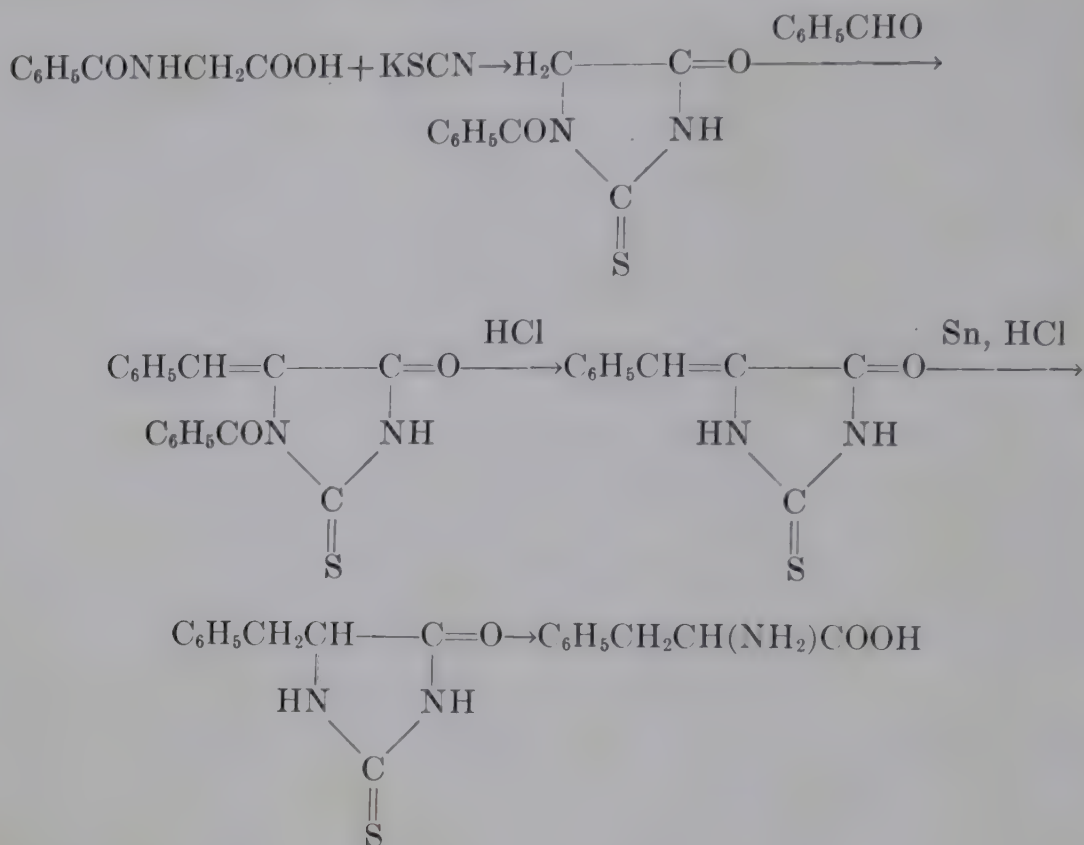


Boyd and Robson (32) found that the initial condensation may be catalyzed by either diethylamine or piperidine in pyridine solution and that the reduction and hydrolysis of the benzalhydantoin may be effected with ammonium sulfide.

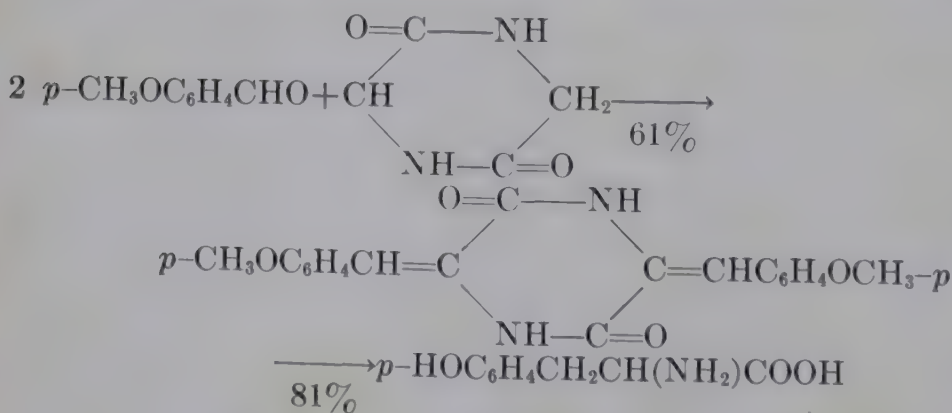
Thiohydantoin may replace the more expensive hydantoin. Thus, Johnson and O'Brien (155) prepared amino acids from aromatic 1-aryl-2-thiohydantoins.

The method has been limited to aromatic amino acids. Aceturic acid gave the corresponding 1-acetyl-2-thiohydantoin.

Sasaki (227) showed that glycine anhydride could undergo a



Perkin reaction with aromatic aldehydes to give the 3,6-diarylidene-2,5-diketopiperazines in good yields. On reduction and hydrolysis the amino acid was formed. DL-Tyrosine was prepared in 50% overall yield. Glycine anhydride has been prepared in 50%

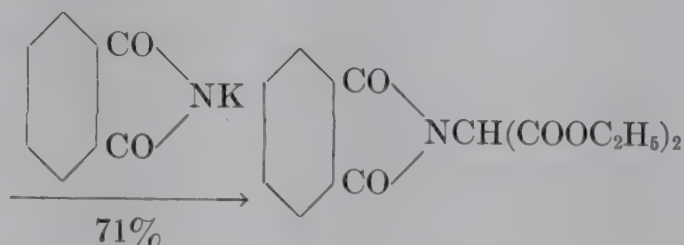
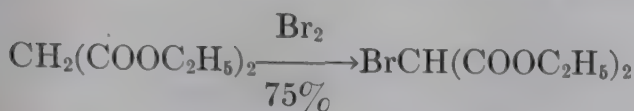


yield by refluxing a solution of glycine in ethylene glycol (226).

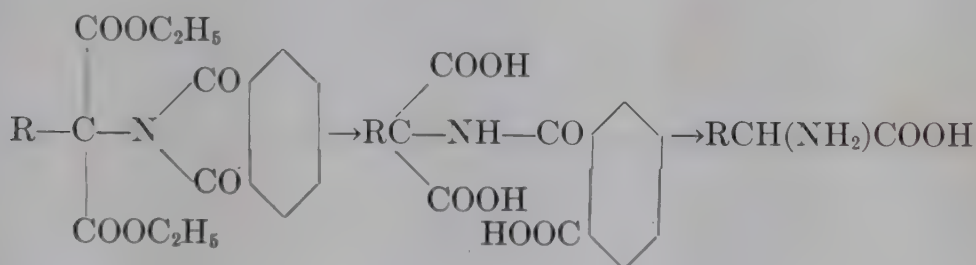
4. Syntheses from ethyl aminomalonate and its derivatives

Ethyl aminomalonate has been used to prepare amino acids, but the instability of the ester and the accompanying N-alkylation militate against its use (182, 215).

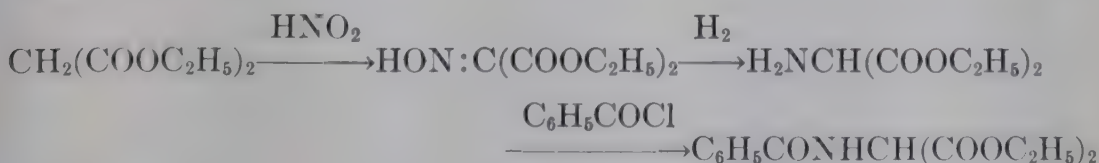
Sørensen (245) protected the nitrogen in ethyl aminomalonate with a phthalyl group. The ester, ethyl phthalimidomalonate, proved to be extremely useful in amino acid synthesis. It was prepared from ethyl bromomalonate and potassium phthalimide (197).



The ester may be alkylated with a variety of alkyl and aralkyl halides and the condensation products then hydrolyzed to the amino acid. In practice, the hydrolysis is usually carried out in two steps.

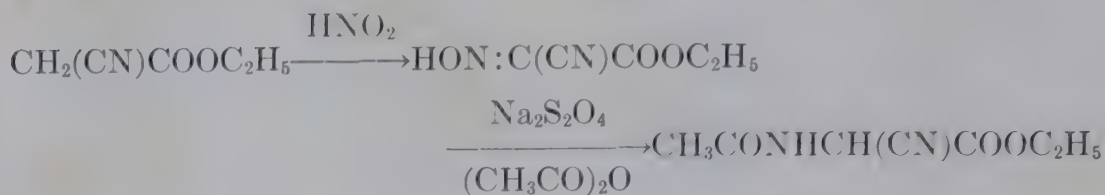


The synthesis may be shortened and the yields improved by using ethyl benzamidomalonate (69). This ester was prepared from ethyl malonate.



Alkylations were carried out with several alkyl and aralkyl halides and chloroesters (219). The alkylated esters were obtained as oils which were hydrolyzed directly with hydrobromic acid. The amino acids were obtained in good overall yield.

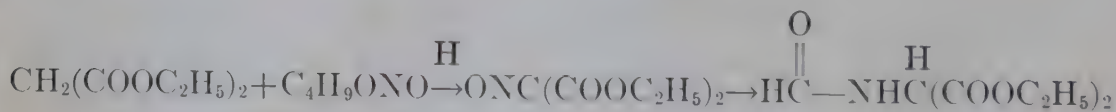
Recently, ethyl acetamidomalonate and ethyl acetamidocyanoacetate were used to prepare amino acids. The former was prepared by Snyder and Smith (244) by acetylation of ethyl aminomalonate with acetic anhydride. Tullar (259) prepared ethyl acetamidocyanoacetate in about 70% yield from ethyl cyanoacetate



without isolation of any intermediates. The alkylation of ethyl acetamidomalonate proceeded smoothly with primary halides (8, 9) but according to Snyder (243) no reaction occurred with secondary bromides. Albertson and Archer (11) found that alkylation with iso-propyl bromide proceeded to the extent of 18%.

Generally, alkylation proceeded better with ethyl acetamidocyanoacetate. Thus, DL-valine (14) was prepared in 43% overall yield with the aid of the cyanoester and DL-isoleucine (15), prepared from secondary-butyl bromide, was obtained in 28% yield. Alkaline hydrolysis of the alkylacetamidocyanoacetates results in complete degradation to the amino acids whereas alkyl acetamidomalonates hydrolyze to the dibasic acid stage only with alkali.

Galat (133a) recommended ethyl formamidomalonate as an intermediate in amino acid synthesis. The preparation of this compound was similar to the one used for the next higher homolog except that reductive acylation was performed with zinc and formic acid.

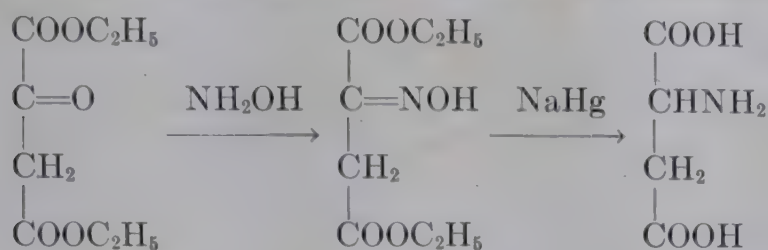


DL-Phenylalanine, DL-aspartic acid and DL-glutamic acid were prepared in 55–60% yield. The advantages of this intermediate are not immediately apparent.

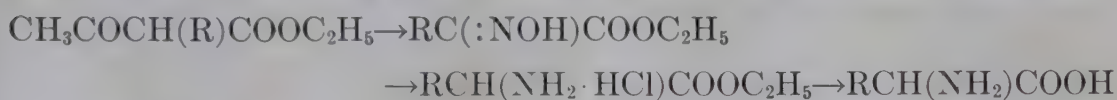
5. The reduction of α -keto acids and their derivatives

Reductive amination of phenylpyruvic acid gave phenacetyl-DL-phenylalanine which hydrolyzed to DL-phenylalanine (92). Similarly, pyruvic acid gave acetyl-DL-alanine (62). Knoop and Oesterlin (164, 165) extended these early observations and found that the reaction could be carried out in the presence of a catalyst. They suggested that the physiological synthesis of amino acids proceeded by reductive amination of α -keto acids.

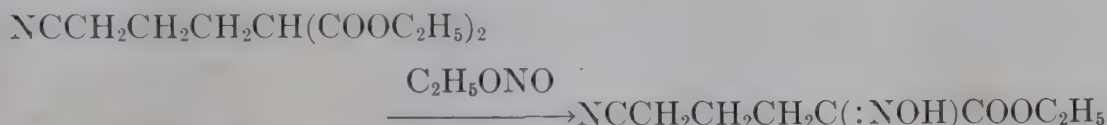
The reduction of α -oximino acids to the corresponding amino acids has been studied extensively. Gutnecht (138) reduced pyruvic acid oxime to DL-alanine and Piutti (212) synthesized DL-aspartic acid from ethyl oximinosuccinate.



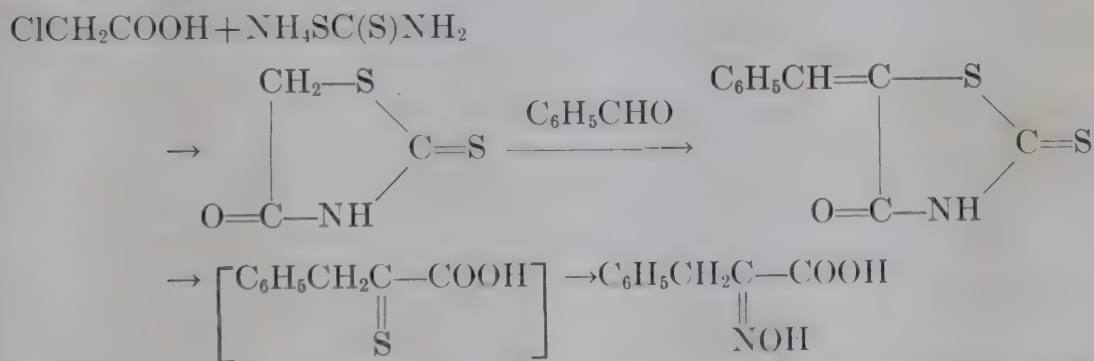
The oxime of phenylpyruvic acid was reduced to DL-phenylalanine by Erlenmeyer (79, 86, 87). The early work was extended by Shemin and Herbst (238) who reduced pyruvic acid oximes catalytically to the corresponding amino acids. α -Oximino acids are best prepared by methods other than by the action of hydroxylamine on substituted pyruvic acids. Bouveault and Locquin (30) and Hamlin and Hartung (139) started from alkylacetoacetic esters.



Fischer (124) used a malonic ester as the source of the oxime in his DL-lysine synthesis.



Gränacher (137) has used the rhodanine synthesis to prepare α -oximino acids.

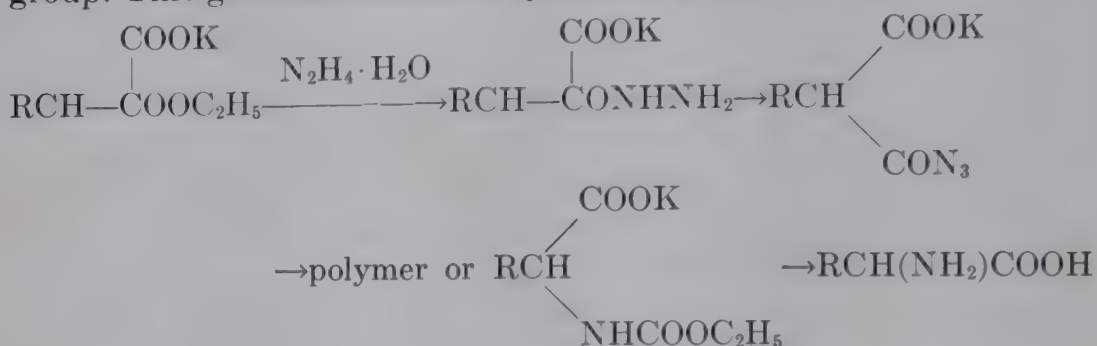


Hydrazones of α -keto acids may be reduced to α -amino acids. Fischer and Groh (114) obtained DL-alanine from pyruvic acid phenylhydrazone. This method has been studied quite extensively by Feofilaktov (100) and his school. Alkylacetoacetic esters are the starting materials. This procedure is reported to give good yields of amino acids.



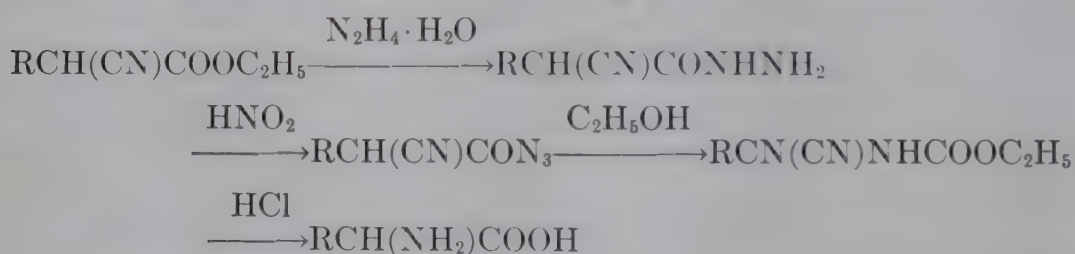
6. Degradative methods

Curtius and Sieber (54, 55, 56) rearranged a half azide of an alkylmalonic acid thereby converting one of the carboxyls to an amino group. This general method of synthesis is illustrated below.

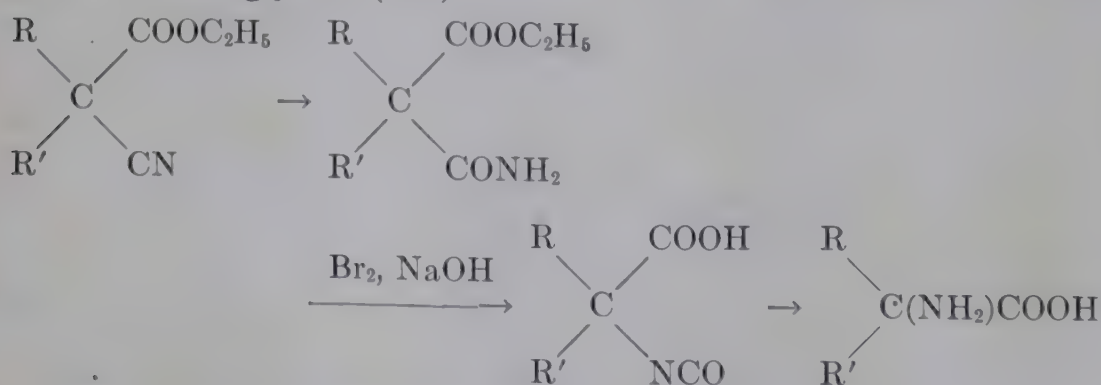


A urethane forms when the rearrangement of the azide is carried out in alcohol.

Darapsky (59, 60) and Gagnon (132) employed a similar synthesis but started with the corresponding alkylcyanoacetic esters.



The Hofman rearrangement has been used to prepare dialkyl derivatives of glycine (177).



Schmidt prepared (232) glycine, DL-phenylalanine and DL-aspartic acid by the action of hydrazoic acid on the proper alkylacetoacetic ester.



Adamson (6) converted α -aminopimelic acid to DL-lysine and α -aminoadipic acid to DL-ornithine with hydrazoic acid and Briggs (36) prepared DL-phenylalanine from benzylmalonic acid by the Schmidt reaction.

None of these degradative methods seem to offer any advantages over the previously discussed synthetic procedures. On the contrary the overall yields are frequently quite low and tedious manipulations are often required to obtain the desired product. In the case of the Schmidt reaction the toxic and explosive nature of the reagent reduces the attractiveness of the method.

7. Addition to double bonds

DL-Aspartic acid has been prepared by the addition of ammonia to maleic and fumaric acids (77). The best preparation of this amino acid has been reported by Dunn and Fox (66) who heated ammonia and diethyl fumarate and hydrolyzed the intermediate diketopiperazine to DL-aspartic acid in about 60% overall yield.

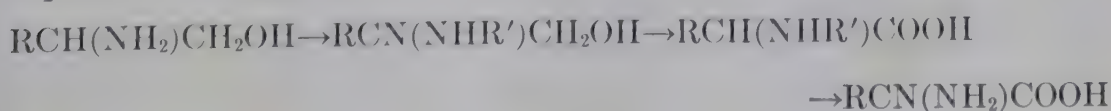
Many syntheses of β -alanine from acrylic acid derivatives are recorded. In one of these, Buc (31) obtained β -alanine in 25% overall yield from acrylonitrile.

DL-Glutamic acid has been prepared from methyl acrylate and ethyl phthalimidomalonate (190), methyl acrylate and ethyl acetamidomalonate (243), and acrylonitrile and ethyl acetamidomalonate (10). The intermediate hydrolyzed to DL-glutamic acid in excellent overall yield.

An interesting synthesis of aromatic amino acids was published recently by Gaudry (134). Acrylonitrile was caused to react with benzenediazonium chloride to give α -chloro- β -phenylpropionitrile, which on hydrolysis and amination gave DL-phenylalanine.

8. Oxidative methods

Billman and Parker (27) prepared amino acids from the corresponding amino alcohols. During the oxidation the amino group was protected with an acyl group. So far only glycine and DL-alanine have been prepared but the method should be applicable to other amino alcohols wherein only the hydroxyl groups are susceptible to oxidation.



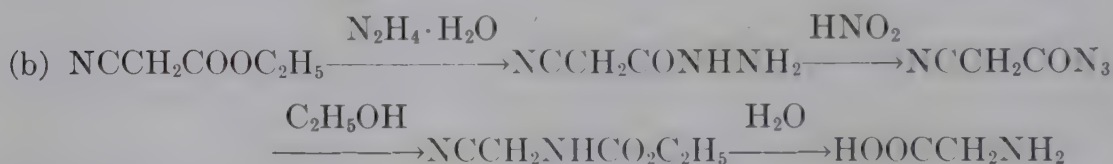
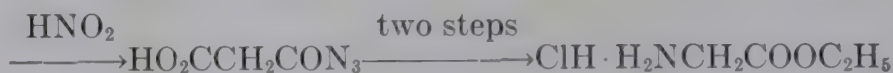
II. Specific Amino Acids

1. Glycine

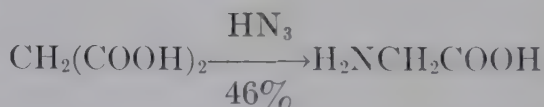
This amino acid has been prepared by a Strecker reaction as noted above. The trimeric methyleneaminoacetonitrile was hydrolyzed with dilute sulfuric acid. The overall yield was 50%.

A better method of preparation was developed by Orten and Hill and later improved by Tobie and Ayres (198). The latter authors obtained glycine in 77% yield from chloroacetic acid. This constitutes the best method for preparing glycine.

Several other preparations have been described, some of which are outlined below. Curtius (55) employed the rearrangement which bears his name. The Darapsky (59, 60) modification of the above method was also used.



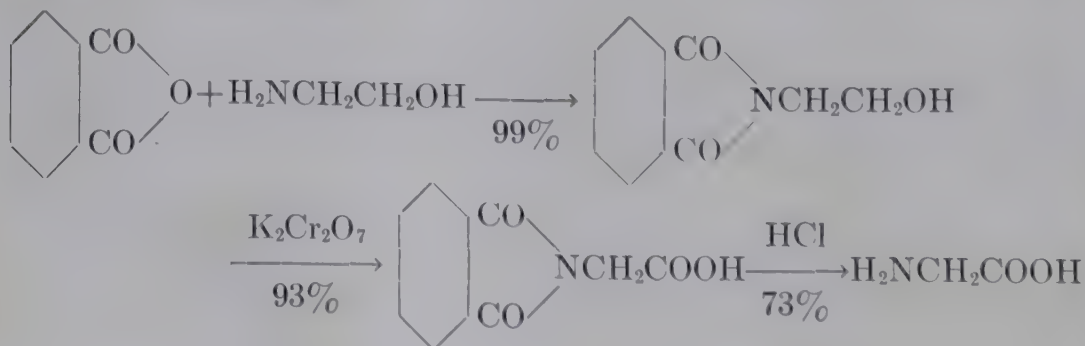
Adamson (6) treated malonic acid with hydrazoic acid and obtained glycine.



The hydrolysis of ethyl benzamidomalonate resulted in the formation of glycine (219).



The preparation of glycine from ethanolamine was studied by Billman and Parker (27). Excellent yields were obtained by their oxidative procedure.



2. DL-Alanine

The preparation from acetaldehyde, sodium cyanide and ammonium chloride gave α -aminopropionitrile which hydrolyzed to DL-alanine in 60% overall yield (195). Better results were obtained with hydrogen cyanide and ammonia, the yield being increased to 72% (53).

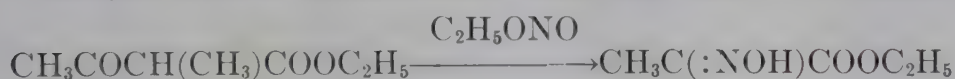
An excellent preparative method has been devised by Tobie and Ayres (256) who aminated α -bromopropionic acid with a large excess of ammonia. They were able to isolate pure DL-alanine in 70% of the theoretical amount.

Redemann and Dunn (219) have methylated ethyl benzamido-malonate and hydrolyzed the resulting product to DL-alanine. This has been confirmed by Painter (210).

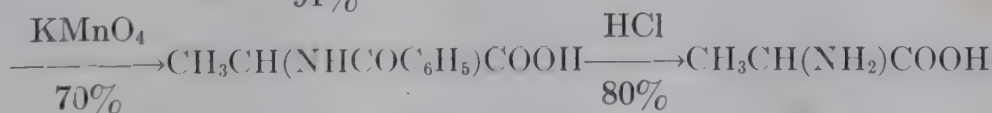
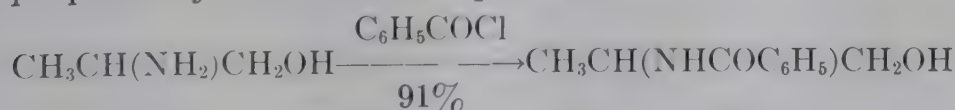
Feofilaktov (104) prepared alanine from ethyl methylacetoacetate.



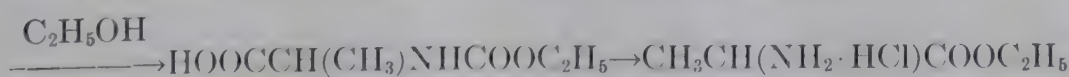
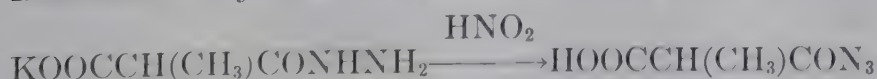
The synthesis of Hamlin and Hartung (139) proceeded similarly.



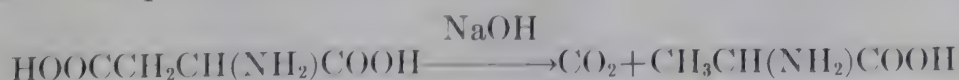
Billman and Parker (27) prepared DL-alanine from 2-amino-1-propanol by their oxidative procedure.



The half-hydrazide of methylmalonic acid has been converted to DL-alanine by Curtius (54) according to the following equations,



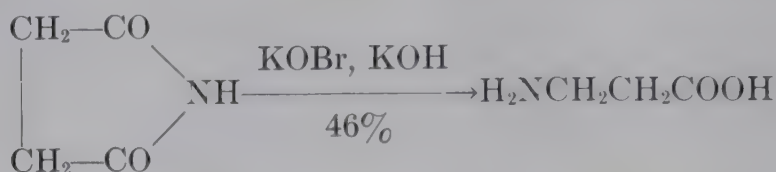
The alkaline degradation of L-aspartic acid yielded DL-alanine as one of the products of the reaction (1).



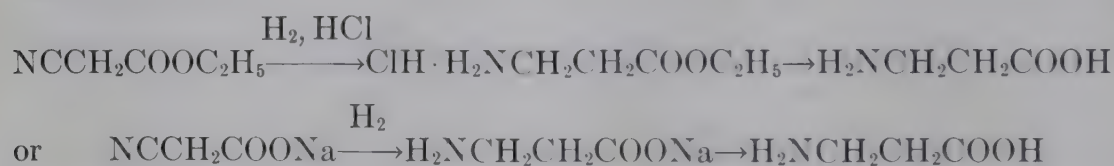
A recent preparation from ethyl acetamidomalonate and cyanoacetate was reported by Albertson (8). Methylation followed by hydrolysis gave the amino acid in unspecified yield.

3. β -Alanine

Although it has been reported that this amino acid is not a constituent of proteins (214) it is of biological interest since it is used as an intermediate in the synthesis of pantothenic acid. β -Alanine was obtained in fair yield by the action of potassium hypobromite on succinimide (203).



Better results have been obtained by the catalytic reduction of esters (264, 224, 159, 230) or salts (37, 20, 225, 40) of cyanoacetic acid.



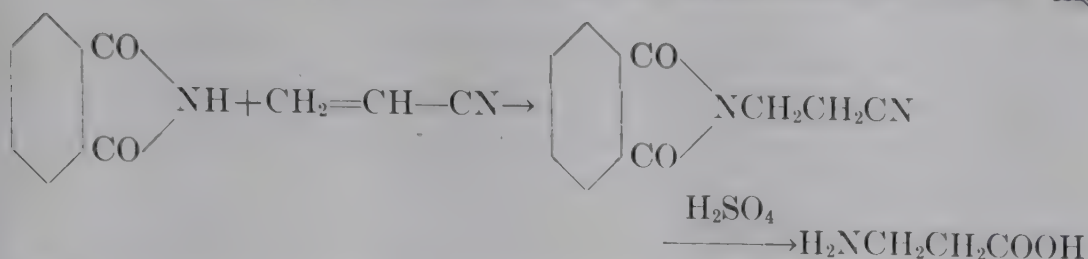
Many studies have been carried out recently on the addition of ammonia to acrylic acid esters and acrylonitrile. In one of these, Buc and co-workers (31) were able to obtain β -aminopropionitrile in 39% yield; the chief by-product was di-(β -cyanoethyl)-amine. Hydrolysis with hydrochloric acid gave β -alanine in about 25% overall yield. A more detailed study (127a) of the amination of acrylonitrile led to the observation that β -aminopropionitrile could be prepared in 60–80% yield.



Alkaline hydrolysis of the nitrile with barium hydroxide gave the amino acid in 90% yield (127). Similar preparations are mentioned in the patent literature (39, 41, 42, 17).

Ethylene cyanohydrin reacted with ammonia at elevated temperature to give β -alanine (61, 163). The methoxyl group in β -methoxypropionitrile may be replaced with an amino group and the product then hydrolyzed to β -alanine in the usual manner (209).

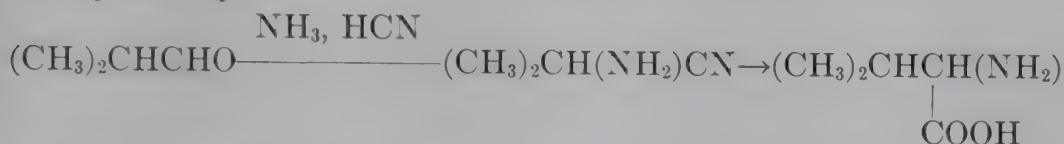
Galat prepared β -alanine from acrylonitrile and phthalimide (133).



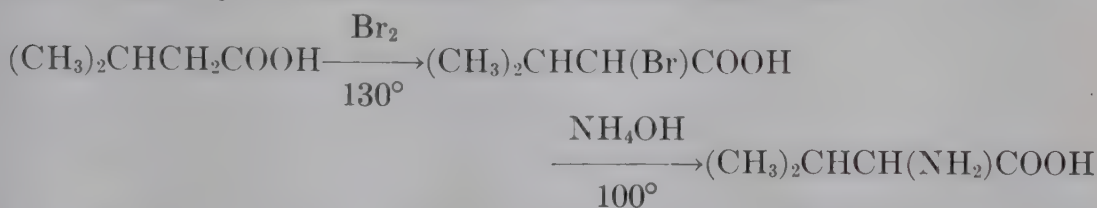
The first step proceeded quantitatively in the presence of benzyl trimethylammonium hydroxide.

4. DL-Valine

Lipp (178, 179) prepared DL-valine by a Strecker reaction from isobutyraldehyde

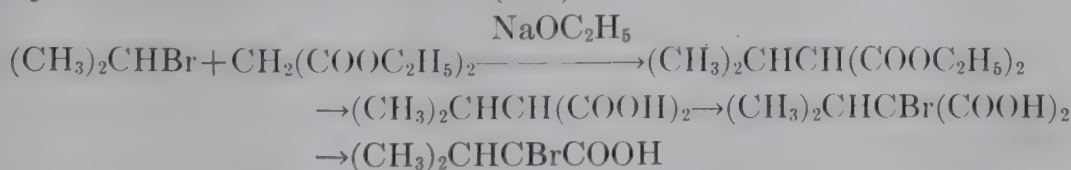


Clark and Fittig (51) apparently were the first to prepare this amino acid by the amination of bromoisovaleric acid.

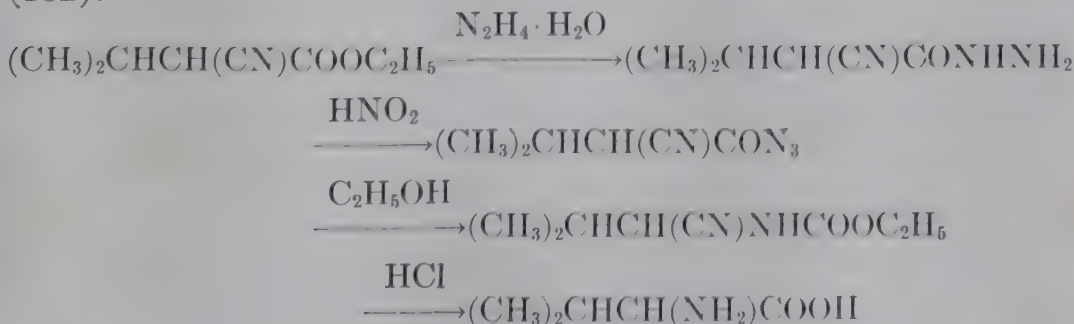


A similar series of reactions were performed by Schmidt and Sachtleben (233). Slimmer (240) aminated α -bromoisovaleric acid with the aid of ammonia and ammonium carbonate, a reaction which was later studied in some detail by Cheronis and Spitzmoeller (50).

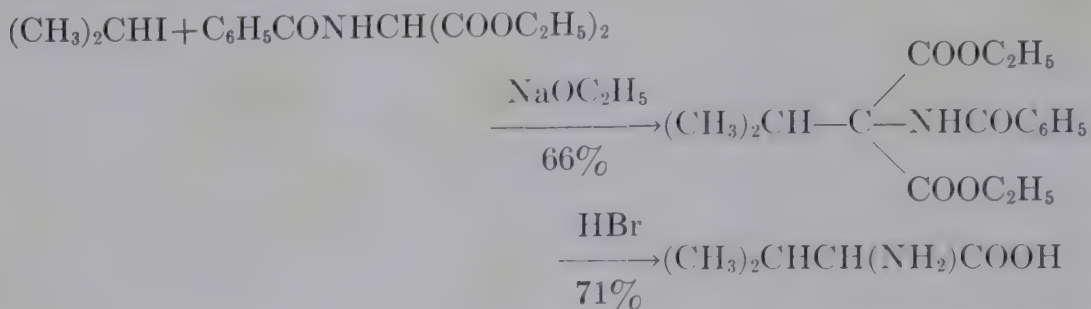
The intermediate α -bromoisovaleric acid has also been prepared by the malonic ester method (204).



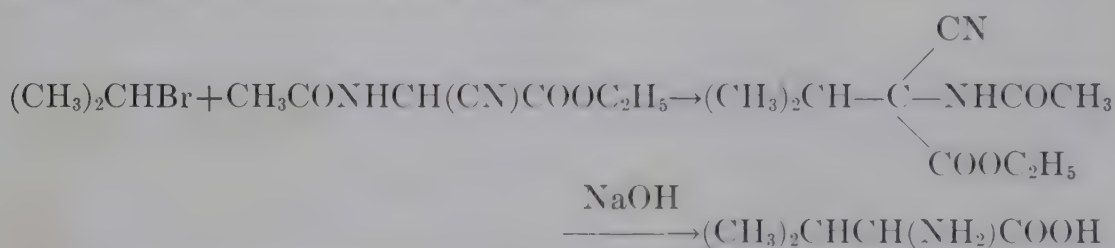
The synthesis of DL-valine from ethyl isopropylecyanoacetate has been carried out by Darapsky (60) and Gagnon and coworkers (132).



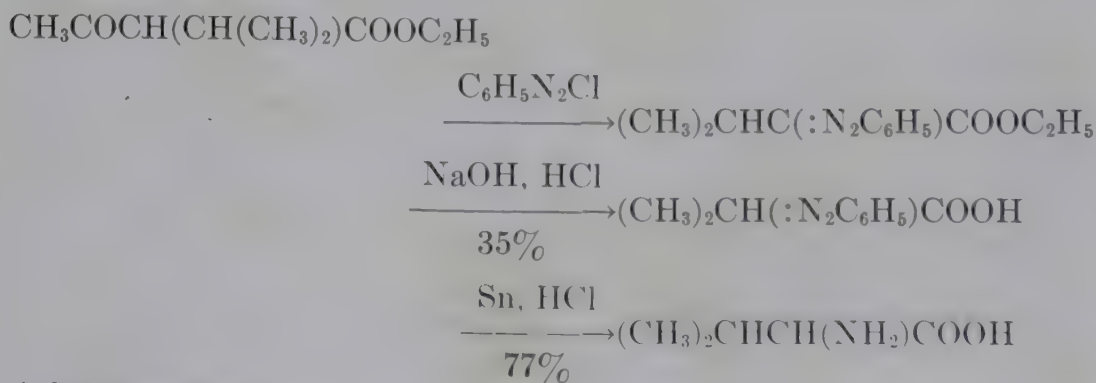
Modifications of the Sørensen synthesis have been employed by Redemann and Dunn (219) and Albertson and Tullar (14). The former group prepared DL-valine in 47% overall yield from ethyl benzamidomalonate and isopropyl iodide.



The amino acid was prepared in somewhat lower yield from ethyl acetamidocyanoacetate and isopropyl bromide.

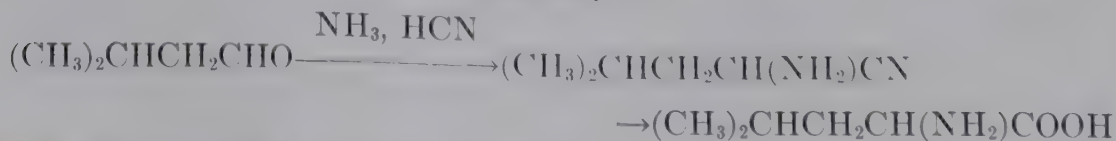


The preparation from ethyl isopropylacetoacetate has been described by Feofilaktov (105).



5. DL-Leucine

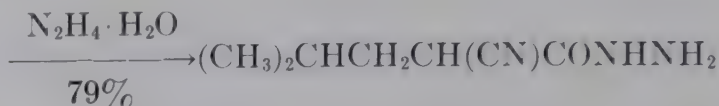
The early syntheses of this amino acid involved the Strecker reaction but it was not clear whether the correct isomer of valeraldehyde was used (176, 252). Hufner (153) specified that his starting material was isovaleraldehyde.



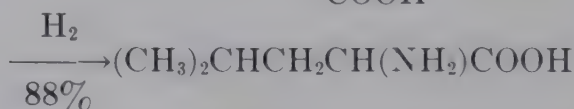
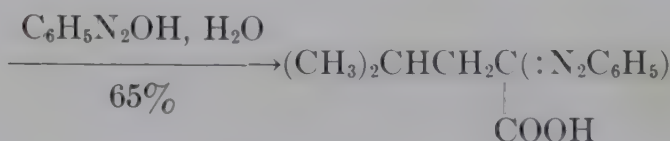
The same author aminated α -bromoisocaproic acid and obtained a substance identical with the one prepared from isovaleraldehyde. Fischer (95) was able to prepare DL-leucine in 70% yield by treat-



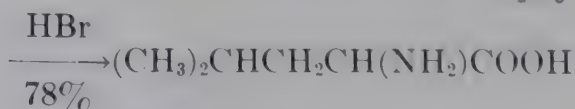
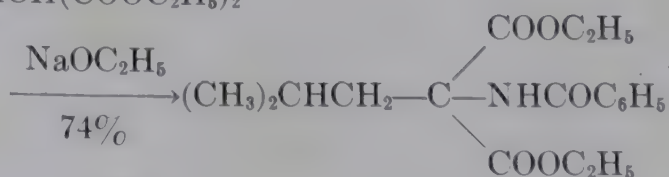
DL-Leucine has been synthesized by Darapsky (59, 60) from ethyl isobutylcyanoacetate.



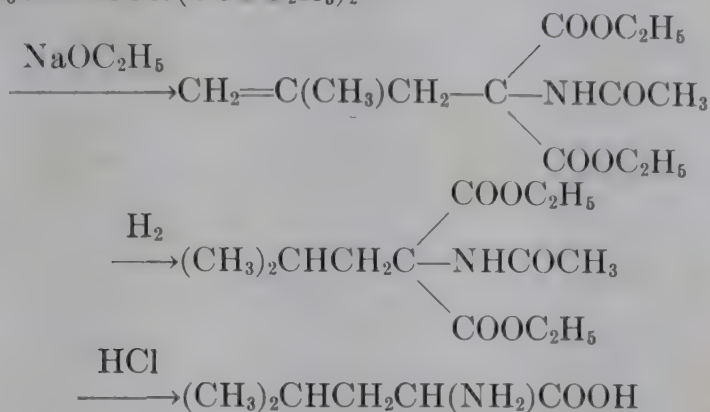
The preparation of DL-leucine from ethyl isobutylacetoacetate has been studied by Feofilaktov who obtained the amino acid in 57% yield from the isobutylated ester (98).



The reaction of isobutyl iodide and ethyl benzamidomalonate proceeded normally to give an oily product which hydrolyzed to DL-leucine (219).



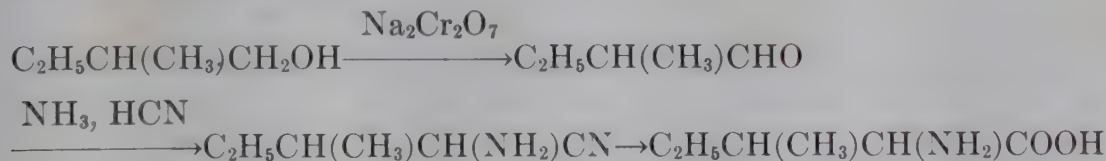
A preparation from the readily available β -methylallyl chloride and ethyl acetamidomalonate was recorded by Albertson and Archer (9).



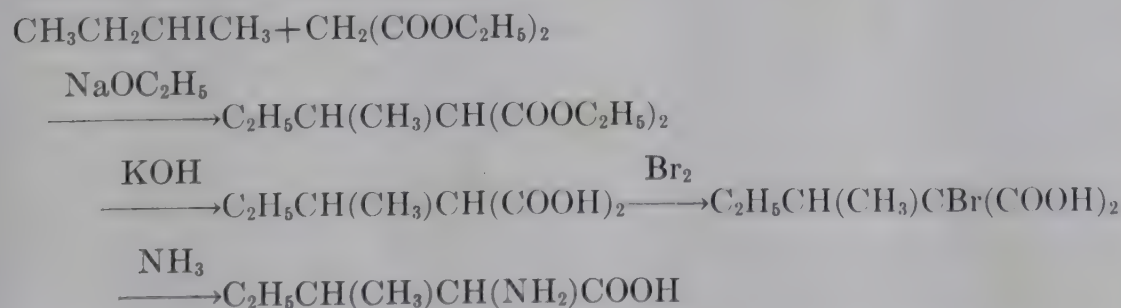
Snyder, Shekelton and Lewis (243) condensed isobutyl bromide with ethyl acetamidomalonate and hydrolyzed the product to acetyl DL-leucine. DL-Leucine has also been synthesized from isobutyl bromide and ethyl acetamidocyanoacetate. The overall yield was 52% (14).

6. DL-Isoleucine

Ehrlich (72) was aware of the presence of two asymmetric carbon atoms in this amino acid. He synthesized a mixture of optically active isomers from active amyl alcohol and destroyed the natural form with yeast (73).



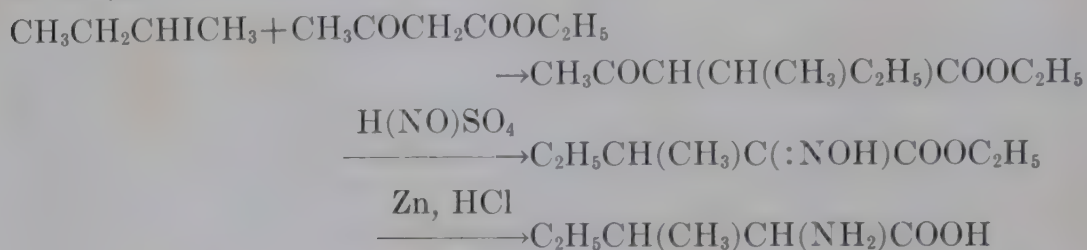
A synthesis of DL-isoleucine from secondary-butyl iodide and ethyl malonate was reported the following year by Ehrlich (74) and Brasch and Friedmann (35).



A detailed preparation, patterned on this method, gave DL-isoleucine in 28% overall yield (188). The preparation from ethyl secondary-butylbromomalonate, the steps of which comprise saponification, decarboxylation and amination was described by Romburgh (223).

It has been demonstrated by Abderhalden and Zeissert (4) that amination of α -bromo- β -methylvaleric acid gives both DL-isoleucine and DL-*allo*isoleucine, the former being less soluble in aqueous ethanol solutions. These authors succeeded in isolating the more soluble isomer from the mother liquors and effecting purification by distillation of the ethyl ester.

DL-Isoleucine has been prepared by reduction of hydrolysis of ethyl β -methyl- α -oximinovalerate which was prepared by Locquin *et al.* (30, 181).



Hamlin and Hartung (139) carried out the nitrosation with ethyl nitrite and reduced the oxime catalytically.

They obtained excellent overall yields with these modifications. Feofilaktov (99) applied his hydrazone synthesis to the preparation of DL-isoleucine. The hydrazone resulting from the action of benzene-diazonium chloride on ethyl secondary-butyldiacetoacetate was reduced to the amino acid. The preparation from secondary-butyl bromide and ethyl acetamidocyanoacetate has been studied (15). The overall yield of DL-isoleucine was 28%.

7. DL-Norleucine

This amino acid is best prepared by the amination of α -bromocaproic acid (2, 5). The yield is about 65%. It has been prepared from ethyl *n*-butylacetoacetate by Hamlin and Hartung (139) and Feofilaktov (101) according to their usual procedures.

Modified Sørensen procedures have also been employed in the synthesis of DL-norleucine. Painter (210) condensed *n*-butyl bromide with ethyl benzamidomalonate and hydrolyzed the intermediate to DL-norleucine. Finally Snyder *et al.* (243) prepared acetyl-DL-norleucine from ethyl *n*-butylacetamidomalonate and Albertson (8) hydrolyzed both ethyl *n*-butylacetamidocyanoacetate and ethyl *n*-butylacetamidomalonate to DL-norleucine in unspecified yield.

8. DL-Norvaline

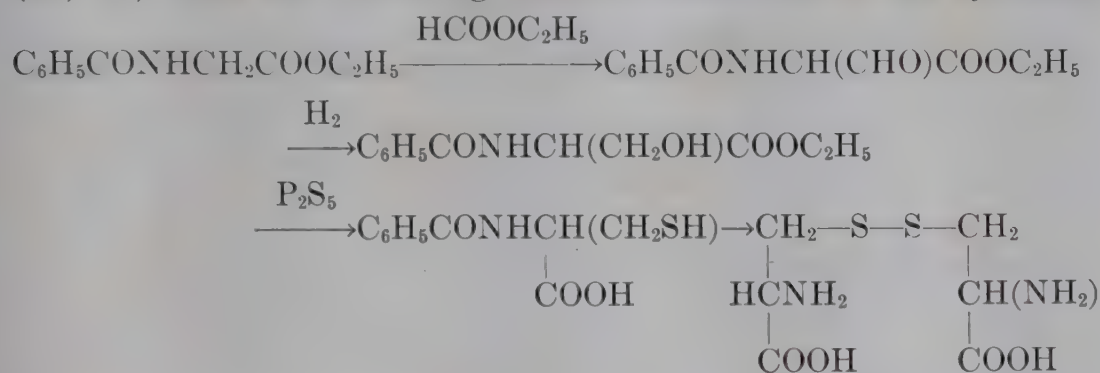
Treatment of *n*-butyraldehyde with ammonium hydroxide and hydrogen cyanide followed by hydrolysis gave DL-norvaline in 68% overall yield (240).

Hamlin and Hartung (139) prepared DL-norvaline from ethyl *n*-propylacetoacetate and Albertson (8) hydrolyzed ethyl *n*-propylacetamidomalonate and ethyl *n*-propylacetamidocyanoacetate to DL-norvaline.

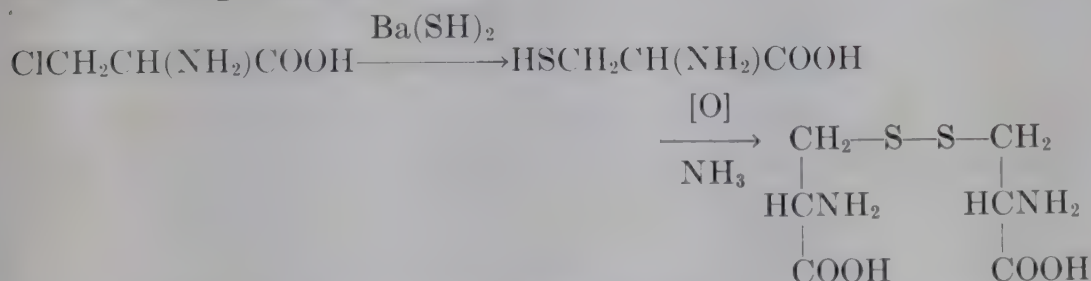
Darapsky (59, 60) converted ethyl *n*-propyl cyanoacetate to DL-norvaline by his method in 31% overall yield.

9. DL-Cystine

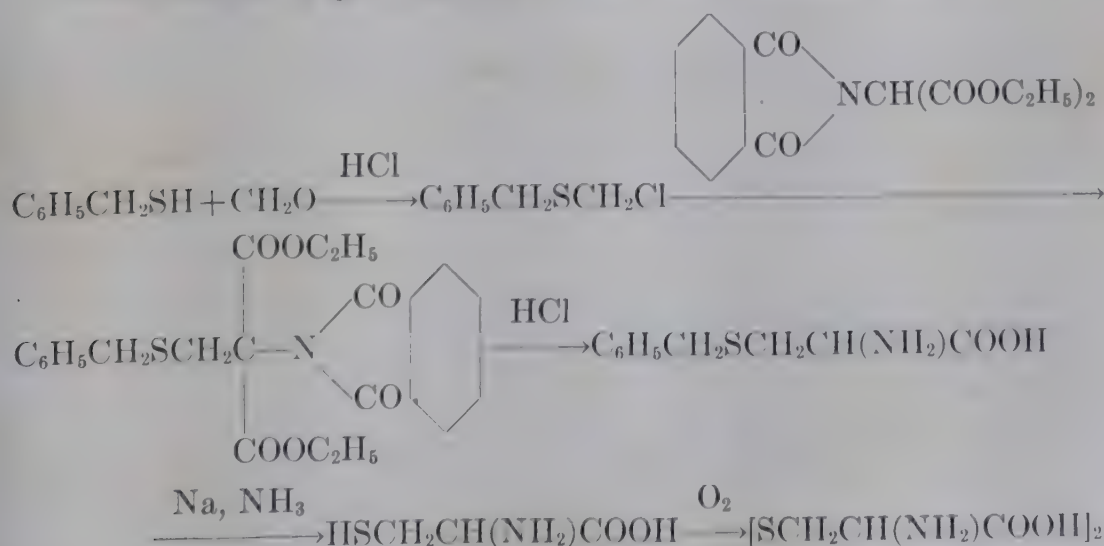
Since this substance can be readily isolated from natural sources little interest has been shown in synthetic methods. Erlenmeyer (89, 97) used the following series of reactions in his synthesis.



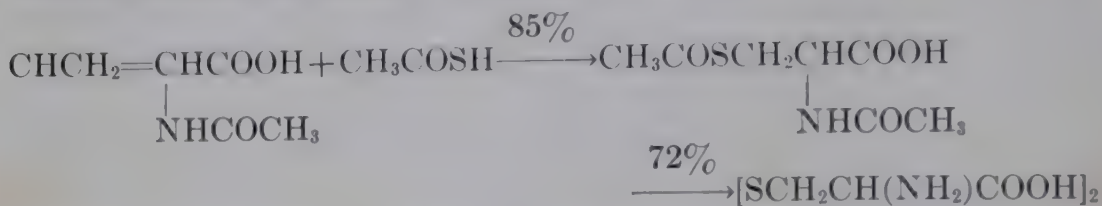
Fischer and Raske (120) used α -amino- β -chloropropionic acid as their starting material.



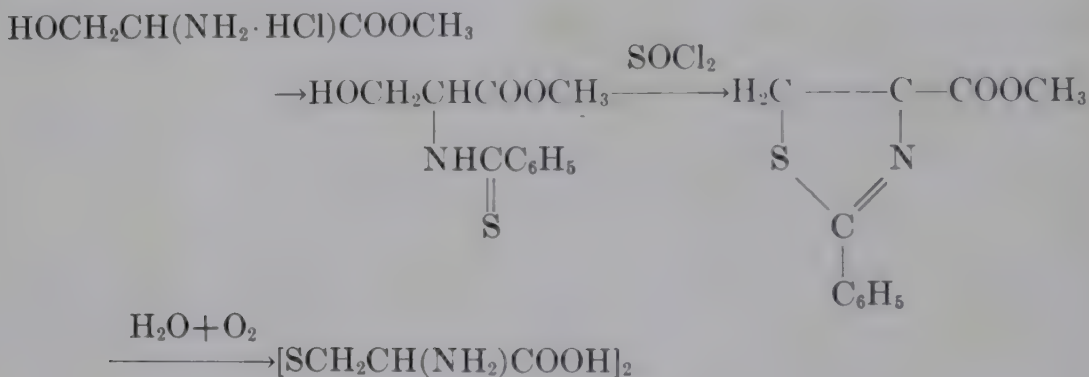
Wood and du Vigneaud (273) used the Sørensen method.



Farlow (97a) prepared DL-cystine in 60% overall yield from α -acetamidoacrylic acid. The steps were:

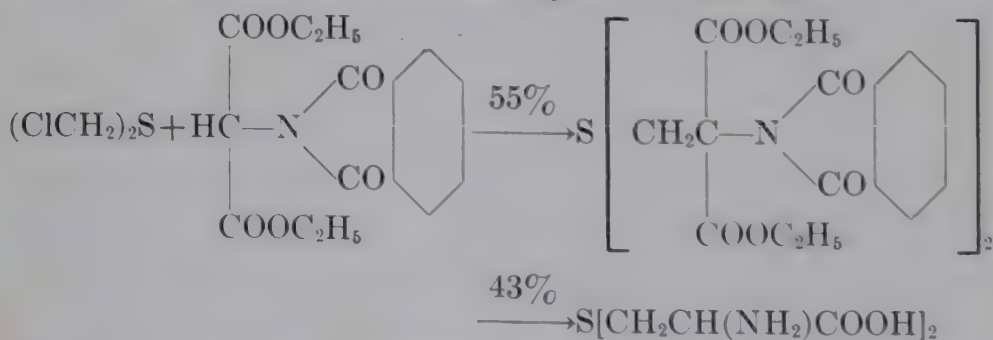


Elliott (76a) converted DL-serine to DL-cystine in the following way.

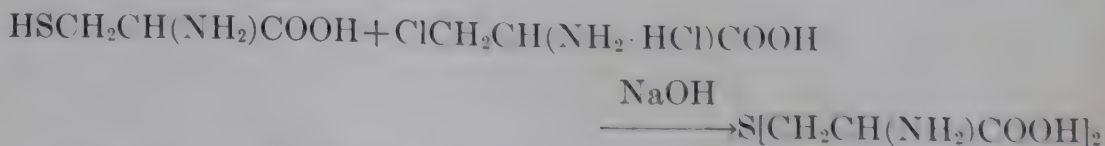


10. DL-Lanthionine

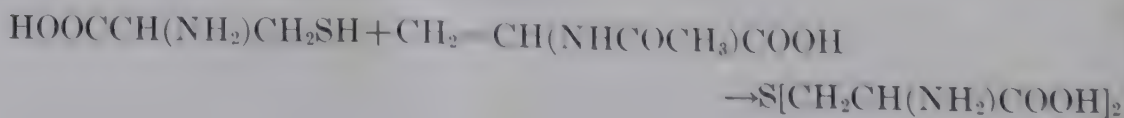
A very similar method was used to prepare this amino acid in 24% overall yield by Kuhn and Quadbach (167).



It had been prepared previously in very poor yield by du Vigneaud and Brown (261).

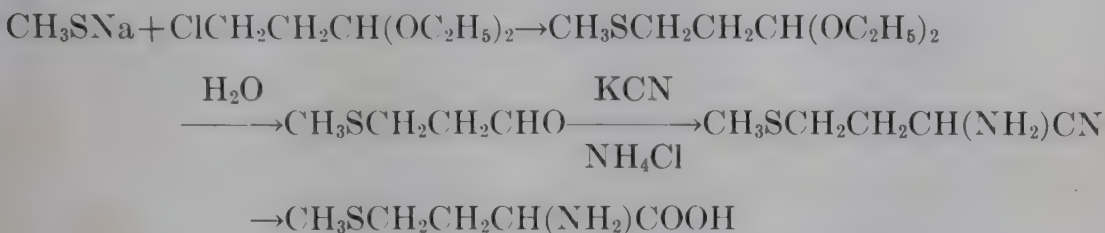


Schöberl (235a) used α -acetamidoacrylic acid in place of chloroalanine hydrochloride and succeeded in obtaining meso-lanthionine in 84% of the theoretical amount.

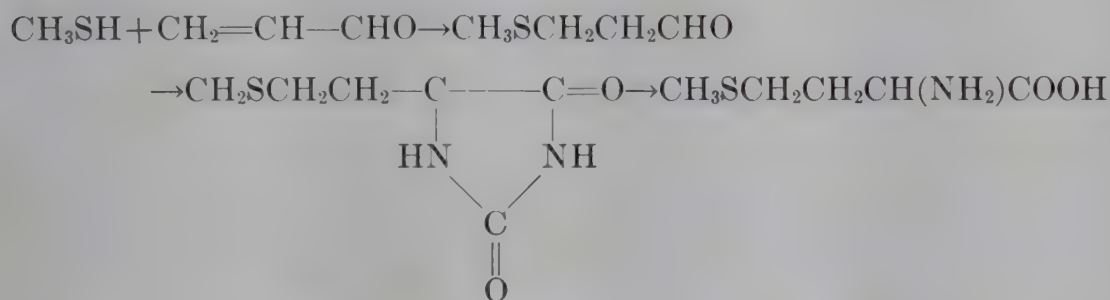


11. DL-Methionine

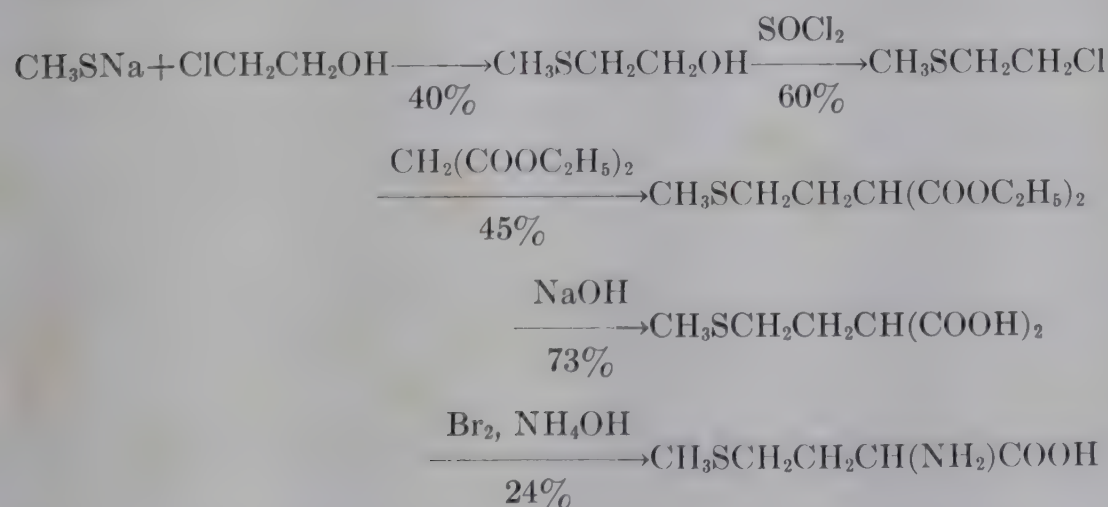
Barger and Coyne (18) prepared this amino acid by a Strecker reaction.



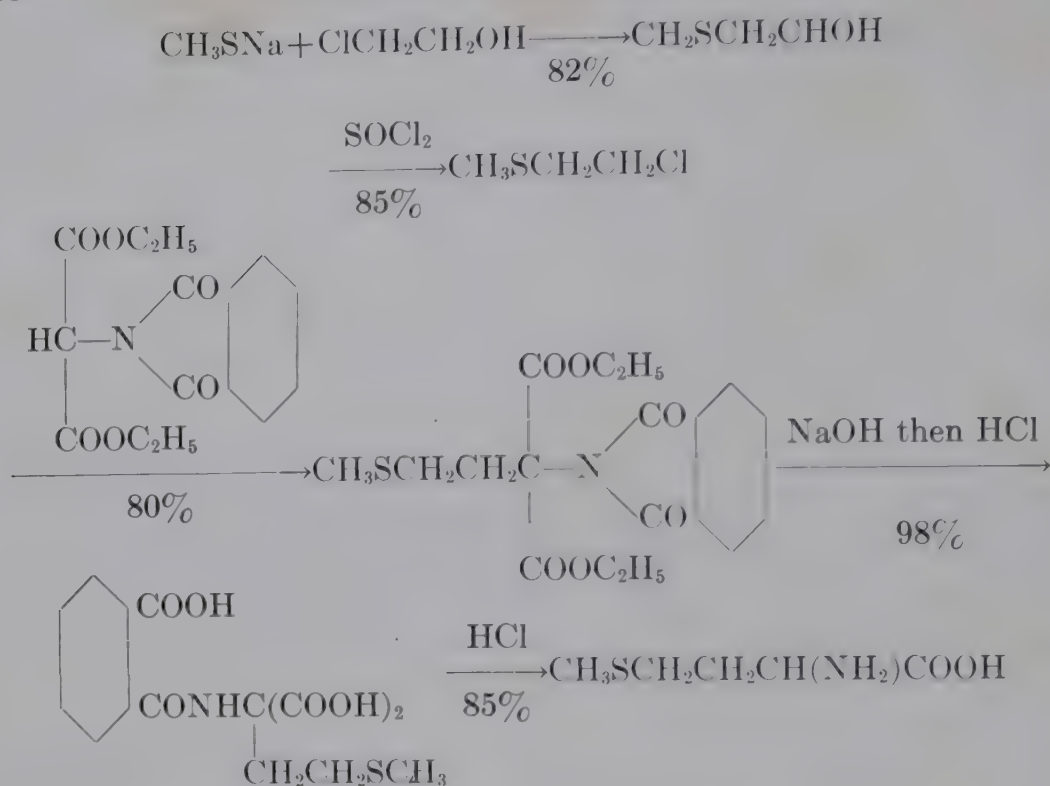
A modification of this method was described by Tishler and co-workers (211d). The methyl mercaptopropionaldehyde was prepared directly from methyl mercaptan and acrolein in 84% yield. The product was converted to the hydantoin which upon hydrolysis gave DL-methionine. The overall yield was 50%.



This is probably the most economical synthesis of this amino acid which has been published to date. The preparation from β -chloroethylmethylsulfide was first undertaken by Windus and Marvel (270).



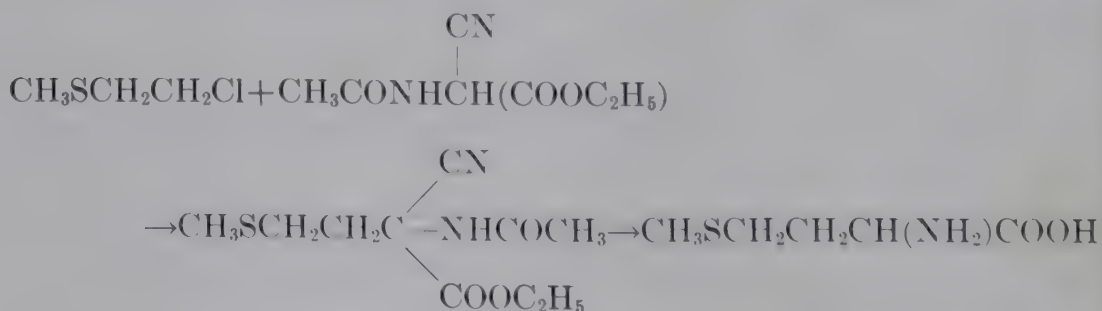
An improved synthesis was reported by Barger (19) who employed the Sørensen method (205). The reactions are:



Some improvements in the early stages of the synthesis have been reported recently (29). The β -chloroethyl methyl sulfide was prepared from β -hydroxyethanethiol in 70–90% yield.

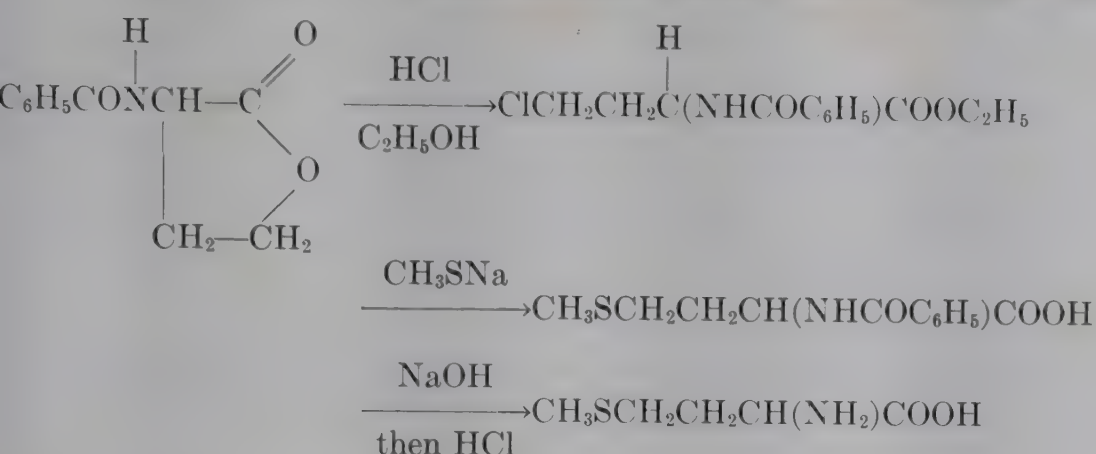


Albertson and Tullar (14) used the same method to obtain this halide but condensed it with ethyl acetamidocyanoacetate. Alkaline hydrolysis gave methionine.

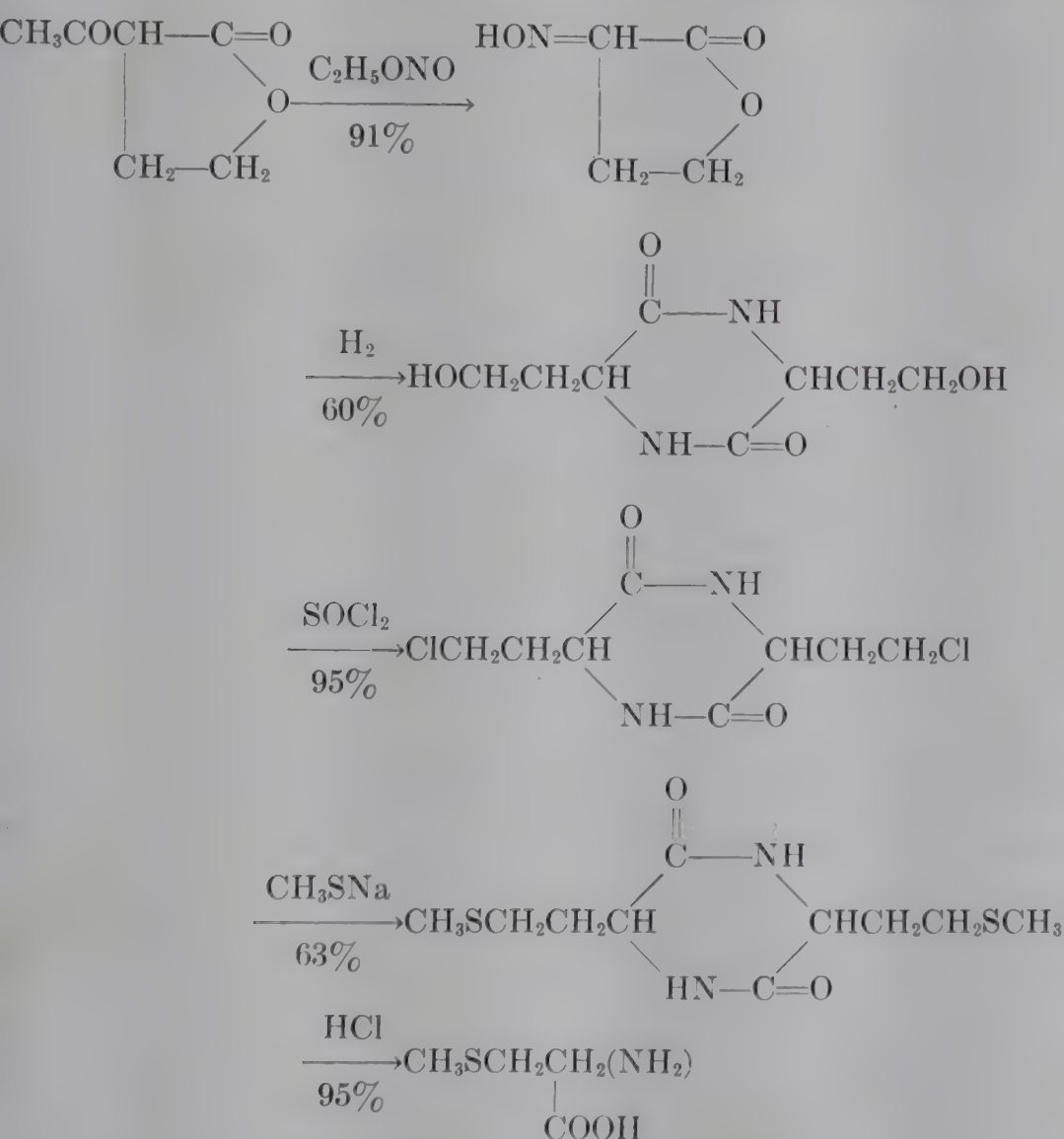


Goldsmith and Tishler (136) showed that ethyl acetamidomalonate may also be employed.

Methods have been devised which avoid the preparation of the vesicant β -chloroethyl methyl sulfide. Hill and Robson (150) prepared methionine according to the following series of reactions.



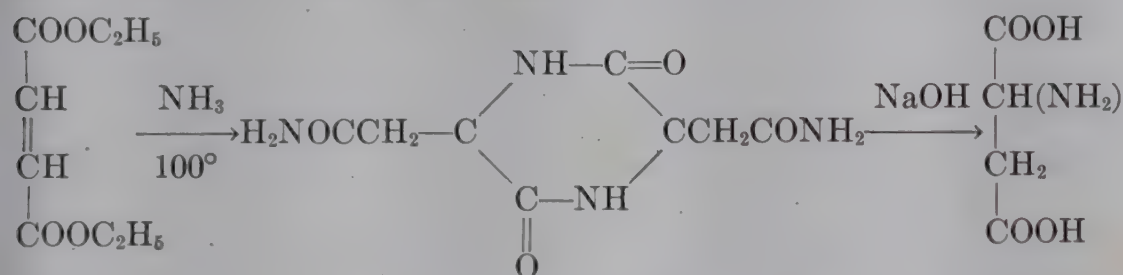
A modification of this synthesis was reported by Snyder (241).



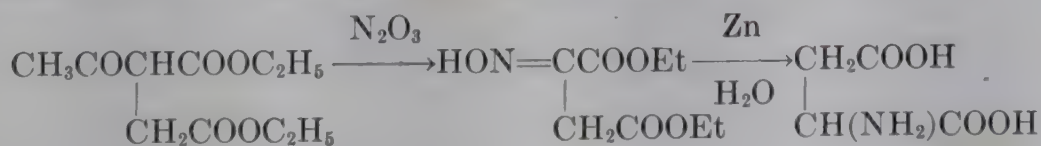
The latter steps in the synthesis have been modified with a concomitant improvement in yield (242).

13. DL-Aspartic acid

The early investigators (63, 77, 78, 271) claimed to have prepared DL-aspartic acid by heating the ammonium salts of maleic, fumaric or malic acids. Heating either maleic or fumaric acids with ammonia resulted in the formation of this amino acid. Koerner and Menozzi (166) succeeded in preparing DL-aspartic acid by the action of ammonia on ethyl fumarate. It was demonstrated later (115) that the initial product in this reaction is diketopiperazinediacetamide. Dunn and Fox (66) found that DL-aspartic acid could be obtained in 59% overall yield from ethyl fumarate and ammonia according to the following equation.

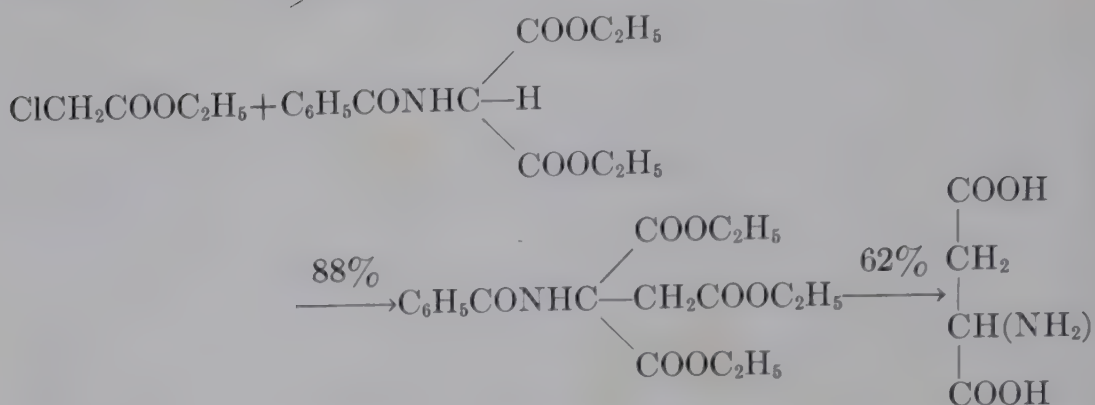


Tutiya (260) reported that fumaric acid may be converted to DL-aspartic acid in 60–65% yield when heated at 180° for 1 hour with two moles of ammonia and four moles of ammonium chloride. This seems to be the most convenient preparation of DL-aspartic acid. By the action of nitrogen trioxide on ethyl acetosuccinate Schmidt and Widman (234) prepared ethyl oximinosuccinate which was reduced and hydrolyzed to DL-aspartic acid.

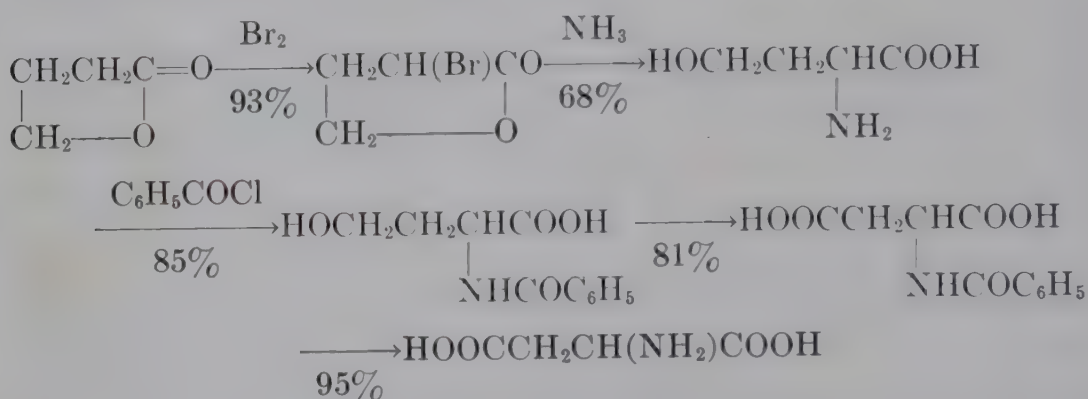


Earlier it was shown by Piutti (212) that ethyl oximinosuccinate may be reduced to the amino acid with sodium amalgam.

DL-Aspartic acid has also been prepared by the alkylation of aminomalonic esters and its acyl derivatives with ethyl chloroacetate followed by hydrolysis. Keimatsu and Kato (160) alkylated ethyl aminomalonate but the DL-aspartic acid was contaminated with aminodiacetic acid which resulted from alkylation of the nitrogen atom. A better synthesis was reported by Dunn and Smart (68) who condensed ethyl phthalimidomalonate with ethyl chloroacetate and hydrolyzed the tri-ester to DL-aspartic acid. Several years later Redemann and Dunn (219) employed the corresponding benzamidomalonate with gratifying results.



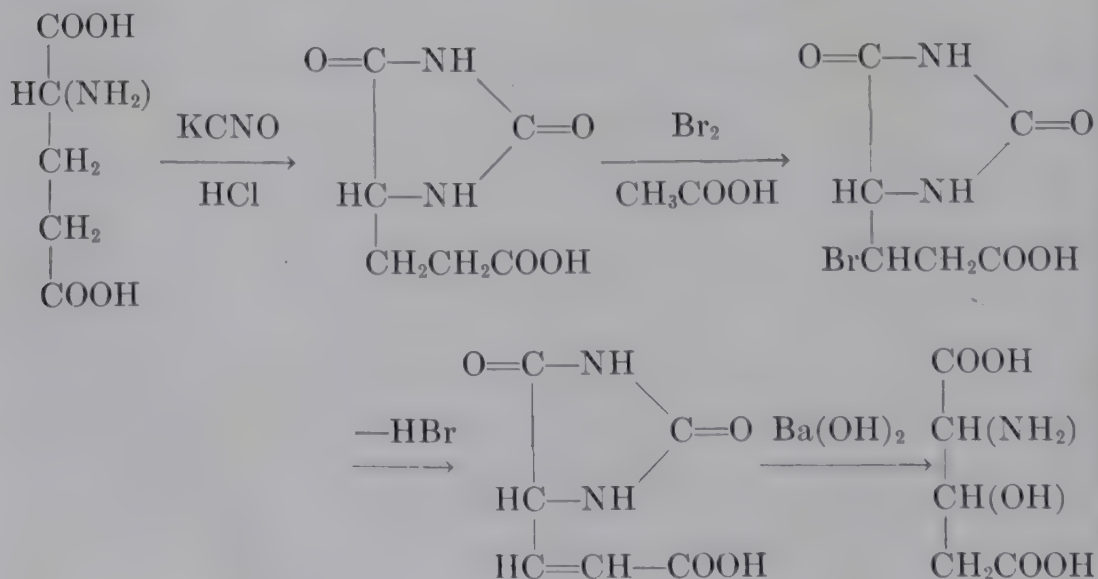
Klosterman and Painter (163a) prepared DL-aspartic acid from butyrolactone in a five stage synthesis.



This synthesis is not nearly as convenient as the method described by Tutiya (260).

14. DL-Hydroxyglutamic acid

Dakin (57) in 1919 reported a synthesis of this amino acid as follows:

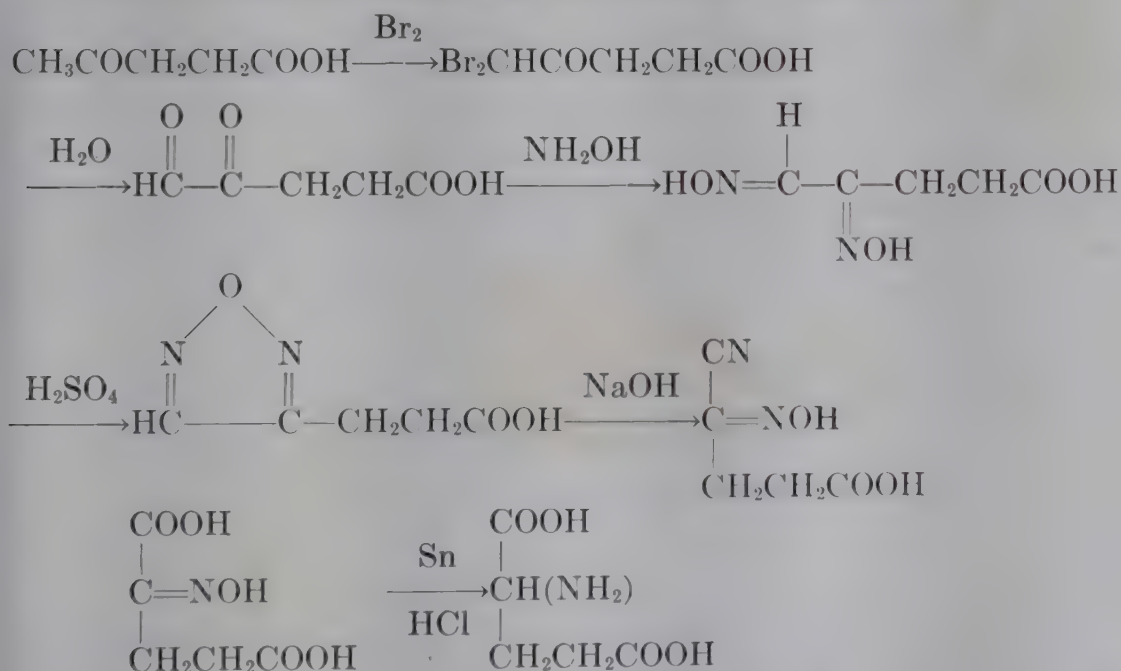


Harington and Randall (145) used a less laborious method to synthesize this acid. Ethyl acetonedicarboxylate was converted to ethyl α -isonitrosoacetonedicarboxylate with the aid of ethyl nitrite. Catalytic reduction of the oximino ester in the presence of a palladium catalyst followed by hydrolysis gave DL-hydroxyglutamic acid.

15. DL-Glutamic acid

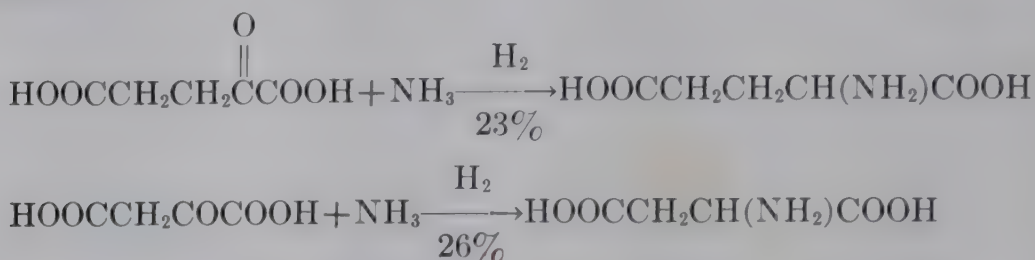
This amino acid is most conveniently prepared from natural sources (200) particularly by the hydrolysis of wheat gluten. The mono-sodium salt, which is commercially available, has been racemized by heating with ammonium chloride followed by refluxing with hydrochloric acid (70). This reaction may be carried out under alkaline conditions (16), or by first converting the amino acid to the DL-pyrrolidone- α -carboxylic acid by simple heating and then hydrolyzing the cyclic amide to the racemic amino acid.

One of the first syntheses was reported by Wolff (272).



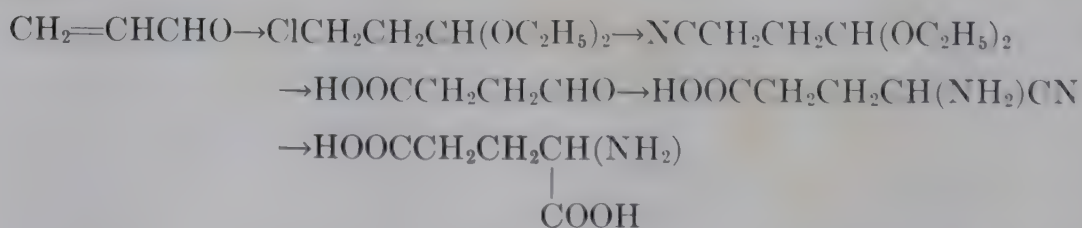
Better methods were developed later for the preparation of α -oximinoglutaric acid or its esters. McIlwain and Richardson (191) cleaved ethyl- α -acetylglutarate with nitrosylsulfuric acid. The oximino ester was reduced and hydrolyzed to glutamic acid in 39% overall yield. Somewhat better results were obtained by Hamlin and Hartung (139) who used butyl nitrite and sulfuric acid to effect the replacement of the acetyl by an oximino group. A palladium catalyst in alcoholic hydrogen chloride served as an effective means of reduction.

Knoop and Oesterlin (164) succeeded in preparing both glutamic and aspartic acids from the corresponding pyruvic acids by hydrogenation in the presence of ammonia with the aid of palladium black.



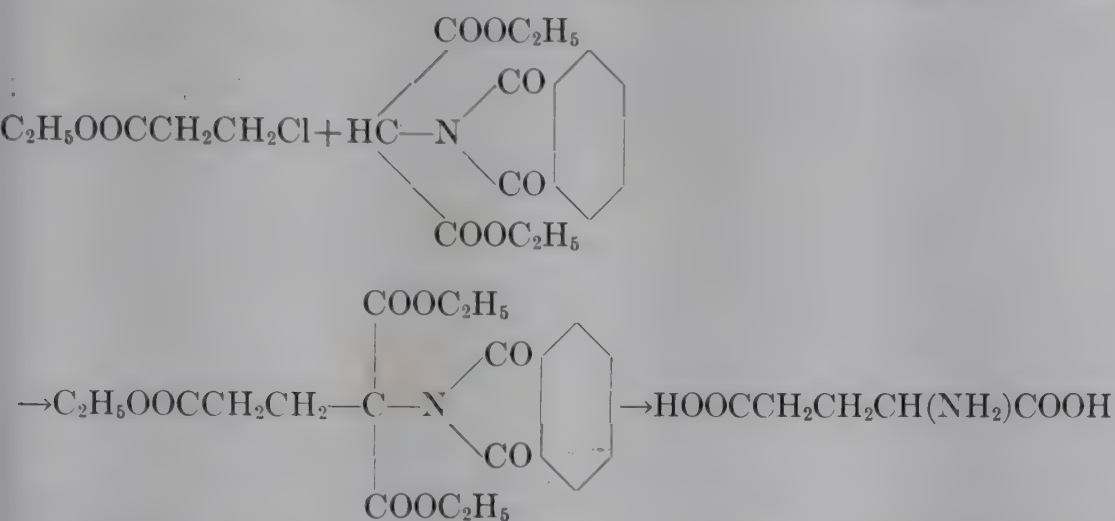
Another reductive method, although quite impractical from a synthetic point of view was studied by Noyes (52) and by Levene (170). The former worker claimed that reduction of optically active ethyl α -diazoglutarate afforded active glutamic acid. Levene repeated this work, but was able to isolate only the racemic amino acid.

A successful application of the Strecker reaction to the synthesis of glutamic acid was reported by Keimatsu and Sugasawa (161). Acrolein was converted to β -chloropropionacetal and thence to β -cyanopropionacetal. Hydrolysis with dilute sulfuric acid resulted in the formation of the corresponding aldehydo acid. The aldehyde was subjected to the usual Strecker reaction and the aminonitrile thus formed saponified to DL-glutamic acid with dilute sulfuric acid.

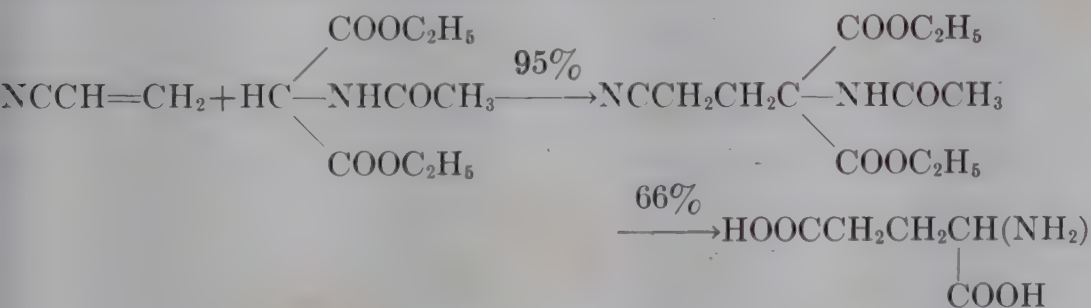


Several syntheses of DL-glutamic acid from substituted aminomalonic esters have been reported. Dunn and coworkers (69, 219) found that ethyl β -bromopropionate condensed with ethyl benzamidomalonate to give ethyl β -carbethoxyethylbenzamidomalonate in 90% yield. Hydrolysis of the ester with hydrochloric acid gave DL-glutamic acid in 52% of the theoretical amount.

Marvel and Stoddard (190) condensed ethyl β -chloropropionate with ethyl phthalimidomalonate and hydrolyzed the resulting product to DL-glutamic acid. Better results were obtained when methyl acrylate replaced the halo ester. The overall yield in this instance was about 80%.

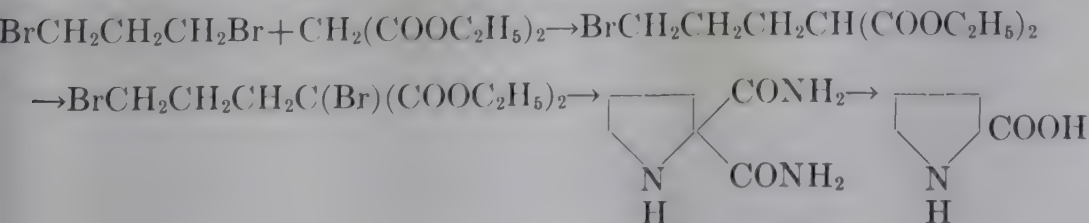


Recently Snyder (243) showed that ethyl acetamidomalonate condensed readily with methyl acrylate to yield a non-crystalline substance, which was hydrolyzed to DL-glutamic acid in 64% overall yield. Albertson and Archer (10) used acrylonitrile instead of the corresponding methyl ester and were able to obtain DL-glutamic acid in approximately the same yield. The cyanoethylation reaction gave almost quantitative yields.

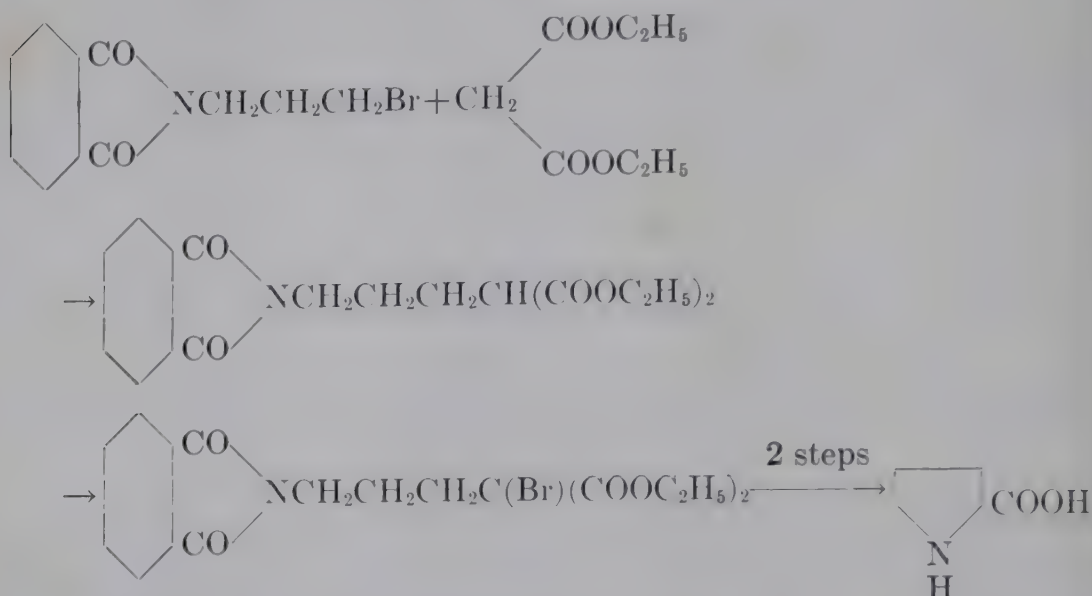


16. DL-Proline

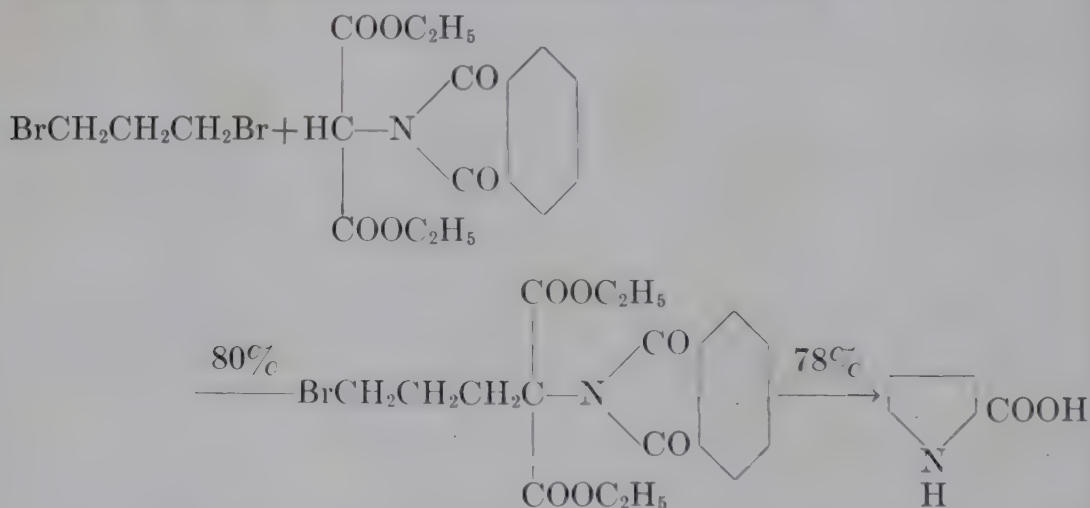
In connection with his studies on the hygrine alkaloids Willstätter (269) synthesized this amino acid from ethyl α -bromo- δ -carbethoxyvalerate according to the following equations.



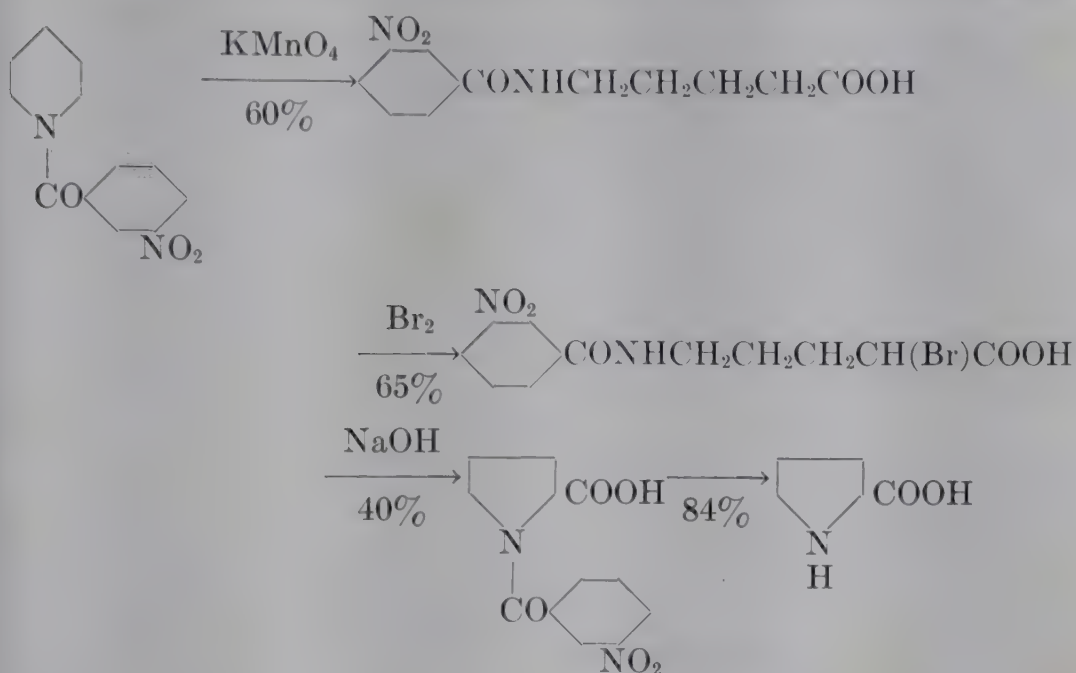
Fischer (107) succeeded in preparing DL-proline from ethyl α -carbethoxy- δ -phthalimidovalerate.



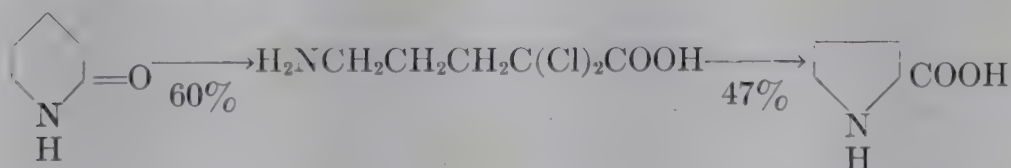
A somewhat similar synthesis was reported by Sørensen (246) who prepared DL-proline from ethyl γ -(bromopropyl)-phthalimidomalonate. Ring closure was effected directly on the bromopropyl ester with the aid of alcoholic sodium hydroxide.



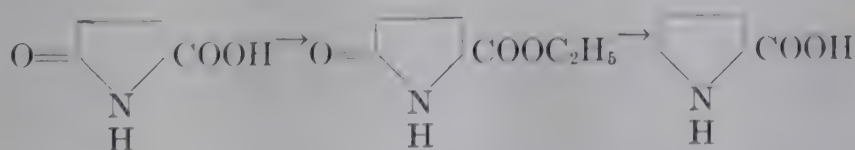
Fischer and Zemlen (125) found that δ -benzamido- α -bromovaleric acid, an intermediate in their ornithine synthesis, underwent cyclization when boiled with dilute hydrochloric acid to give proline in 65% yield. In a later paper the authors recorded some improvements in the synthesis (126). The oxidation of *m*-nitrobenzoylpiperidine proceeded in better yield than the same reaction with benzoylpiperidine. The ring closure of the bromo acid was carried out in dilute alkaline solution to yield *m*-nitrobenzoyl-DL-proline. This substance was resolved before hydrolysis. Later studies (235) were made on the preparation of some of the intermediates.



An interesting synthesis from α -piperidone was reported by Heymons (149). Chlorination gave δ -amino- α,α -dichlorovaleric acid which on treatment with sodium amalgam underwent reduction and spontaneous ring closure to DL-proline. The overall yield was about 30%.

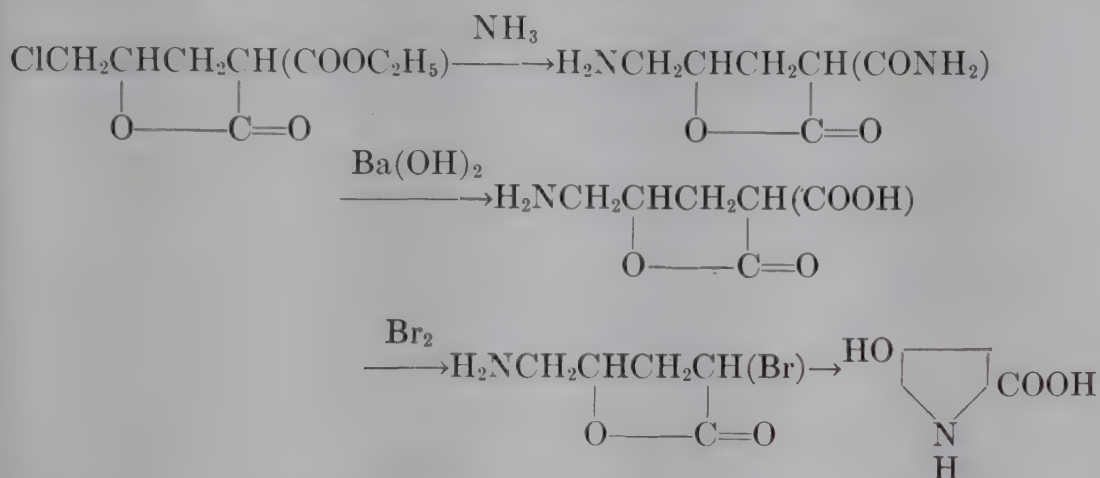


An apparently attractive synthesis of DL-proline would be by the reduction of ethyl pyrrolidone- α -carboxylate which may be prepared from D-glutamic acid. Fischer and Bochner (112) were able to prepare the ester in only 41% yield and the subsequent reduction did not proceed smoothly.

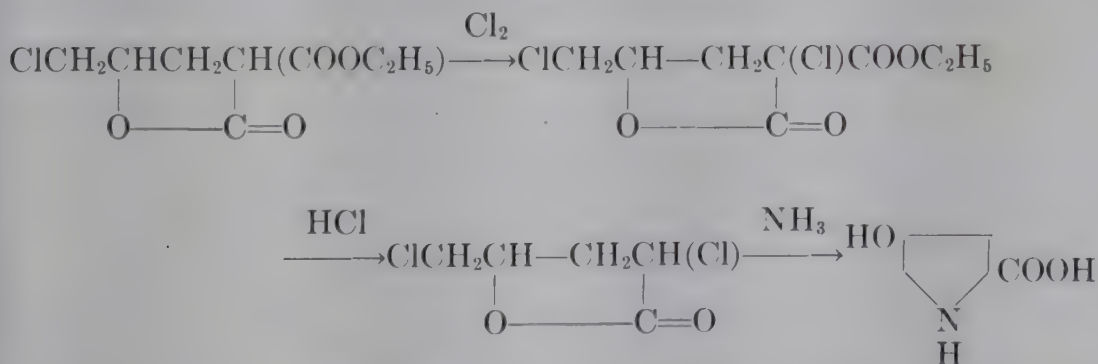


Attempts have been made to prepare DL-proline from pyrrole (215). Signaigo and Adkins (239) obtained the amino acid in about 55–60% overall yield according to the following scheme. This is probably the most convenient preparation available for this amino acid.

Both Traube (257) and Leuchs reexamined the synthesis of DL-hydroxyproline from α -carbethoxy- δ -chlorovalerolactone. The former treated the lactone with ammonium hydroxide to obtain first, δ -amino- α -carbamidovalerolactone which was hydrolyzed to a non-crystalline acid, brominated and cyclized to give one of the diastomeric DL-hydroxyprolines in poor overall yield.

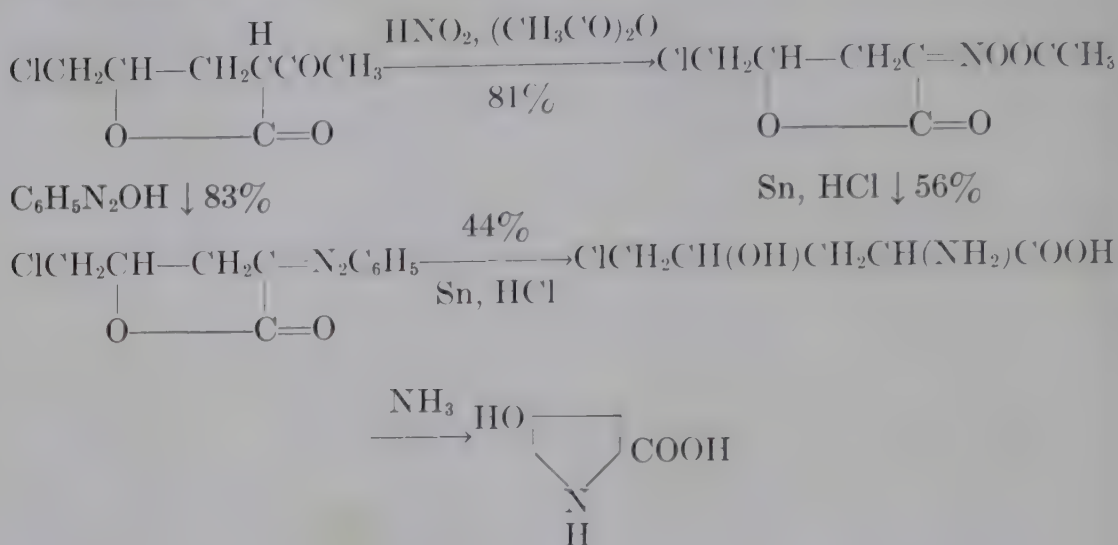


Leuchs (175) subjected the initial lactone to chlorination and isolated two isomeric dichlorolactones. These were hydrolyzed and decarboxylated separately to yield isomeric dichlorovalerolactones which were then aminated. Both lactones gave the two isomeric hydroxyprolines indicating that a partial Walden inversion had occurred either in the decarboxylation or amination steps or both.



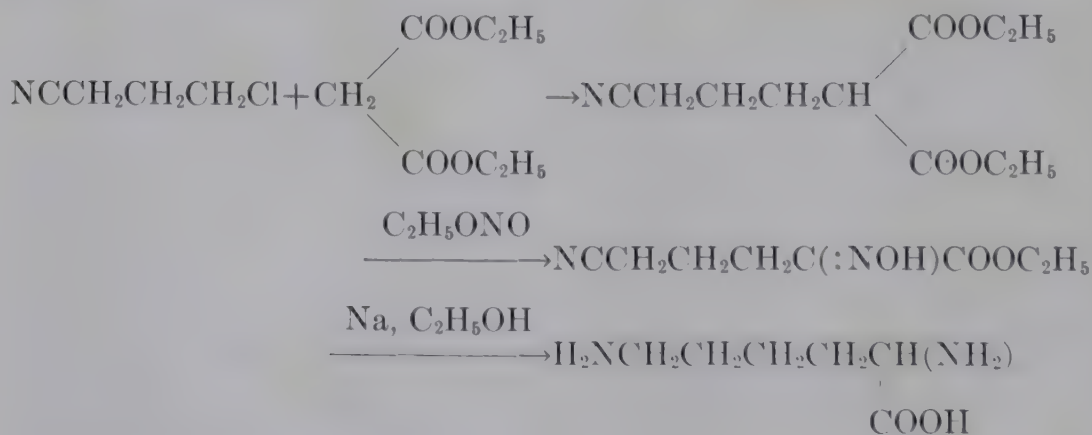
In more recent years Feofilaktov (102) synthesized this amino acid by a somewhat different procedure. α -Aceto- δ -chlorovalerolactone which had been prepared previously by Traube and Lehman (258) from epichlorohydrin and ethyl acetoacetate was converted to the oxime acetate and phenylhydrazone of δ -chlorovalerolactone with nitrous acid-acetic anhydride and benzenediazonium chloride, respectively. Both lactones were reduced and hydrolyzed to a mixture of racemates of α -amino- γ -hydroxy- δ -chlorovaleric acid. The

less soluble isomer was converted to the β -form of DL-hydroxyproline, whilst the more soluble form gave the α -form on amination.

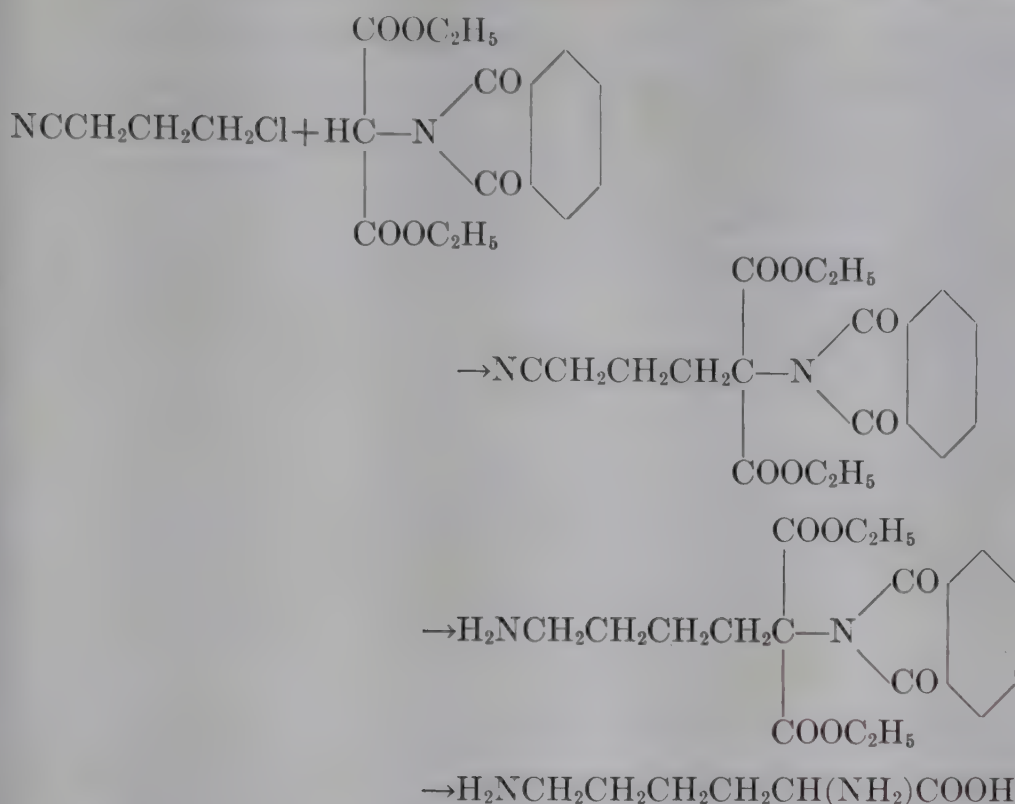


18. DL-Lysine

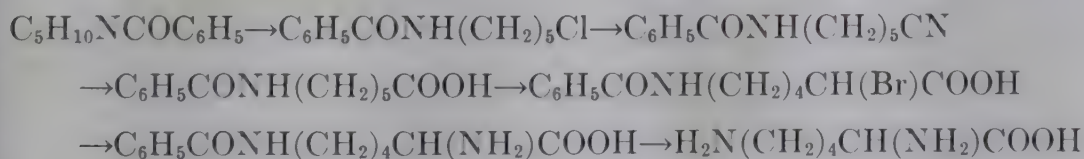
A successful synthesis of this amino acid was reported by Fischer and Weigert in 1902 (124). Ethyl malonate was condensed with γ -chlorobutyronitrile to give ethyl γ -cyanopropylmalonate. On treatment with ethyl nitrite an oximino group replaced one of the carbethoxyl radicals. The resulting α -nitroso- δ -cyanovaleric ester was reduced with sodium and alcohol to give lysine in rather poor overall yield.



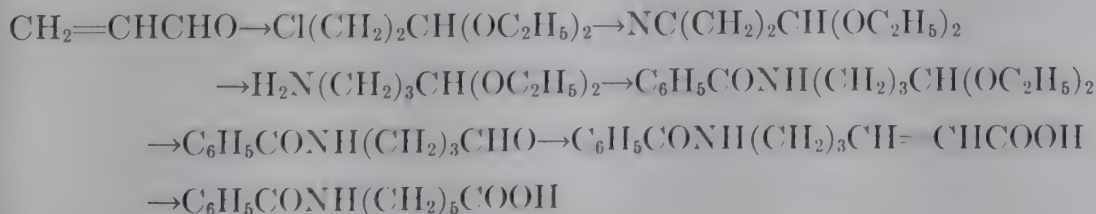
Another method which used γ -chlorobutyronitrile was reported by Sørensen (245). Ethyl phthalimidomalonate was caused to react with the cyano-halide and the condensation product was then reduced. Hydrolysis and decarboxylation afforded the amino acid.



Von Braun (34) used a different route to prepare this amino acid. Benzoylpiperidine was converted to ϵ -benzamidopentylchloride, this in turn was transformed to ϵ -benzamidocaproic acid through the intermediate nitrile. Amination of the bromo acid resulting from the action of bromine and phosphorus on the benzamido acid led to benzoyl-DL-lysine which was readily hydrolyzed to the dibasic amino acid.

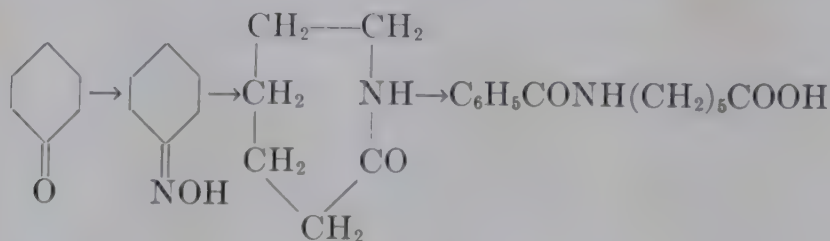


A less useful synthesis of ϵ -benzamidocaproic acid was published by Sugawara (253), as shown in the following equations.



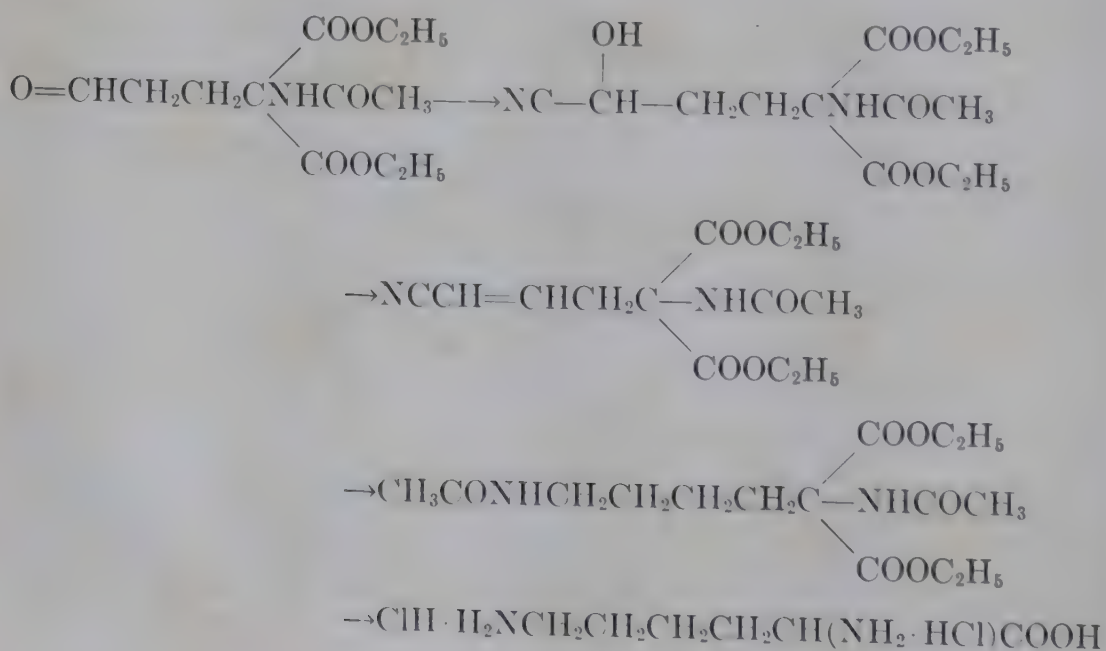
The best method for the preparation of the benzamidocaproic acid and also DL-lysine was described by Eck and Marvel (71). Cyclohexanone was converted to its oxime which was subjected to a

Beckmann rearrangement. The lactone was hydrolyzed and benzo-ylated to give the caproic acid. The method of von Braun was then followed to complete the synthesis. An overall yield of 22–23% was claimed in the original paper but the method described later gave better results with yields of 28%. Maeda and Nozoe (184) stated that they were able to increase the yield of DL-lysine to 50% based on cyclohexanone. Recently the cyclic lactam has become available commercially, so that the most troublesome step has been effectively eliminated.



Galat (133b) has improved the yield in the halogenation step. Chlorination of ϵ -benzamidocaproic acid with sulfuryl chloride in the presence of catalytic amounts of iodine resulted in the formation of the α -chloro acid in 97.5% yield. Amination of the crude acid gave monobenzoyl-DL-lysine in 60% yield.

A new approach to this amino acid was recorded by Warner and Moe (262a). The condensation product from acrolein and ethyl acetamidomalonate was converted to the cyanohydrin which in turn was dehydrated and reductively acylated in 52% overall yield. Hydrolysis and decarboxylation gave DL-lysine hydrochloride in 77% yield.

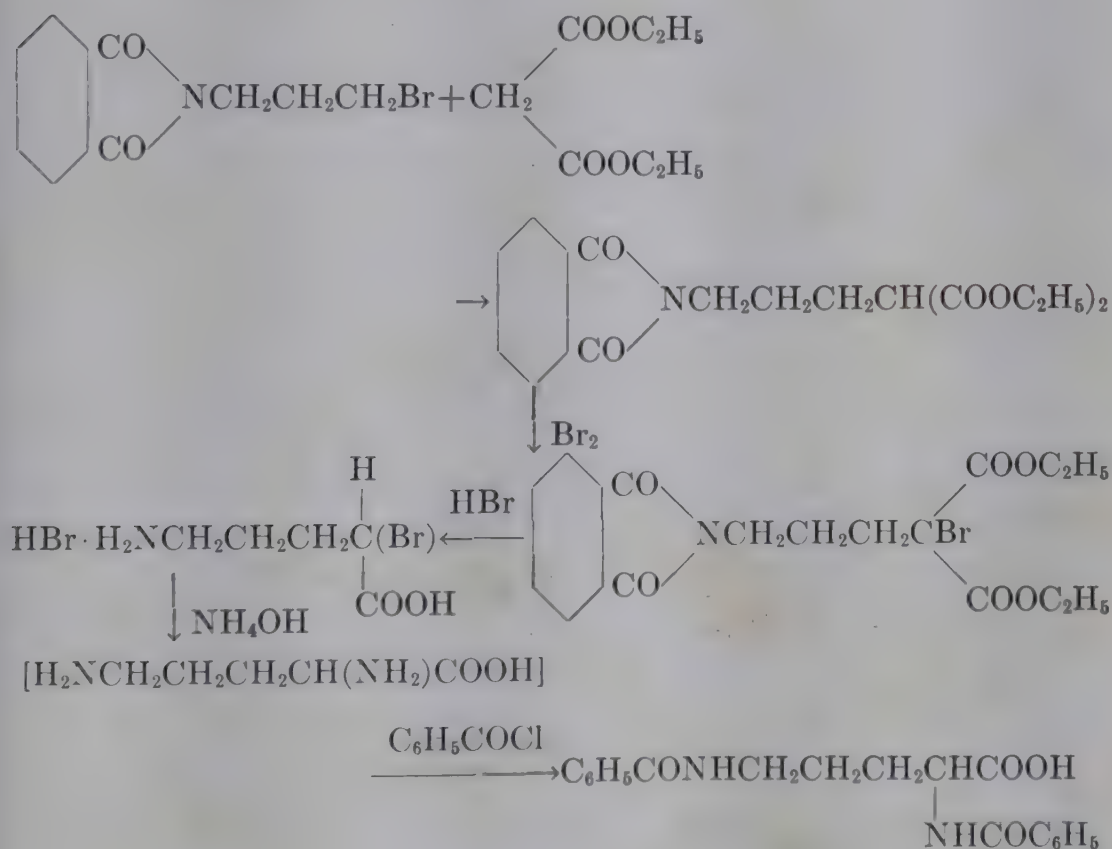


An interesting but rather impractical synthesis of lysine was reported by Adamson (6). He found that hydrazoic acid reacted preferentially with the distal carboxyl in α -aminopimelic acid thereby converting it to an amino group.

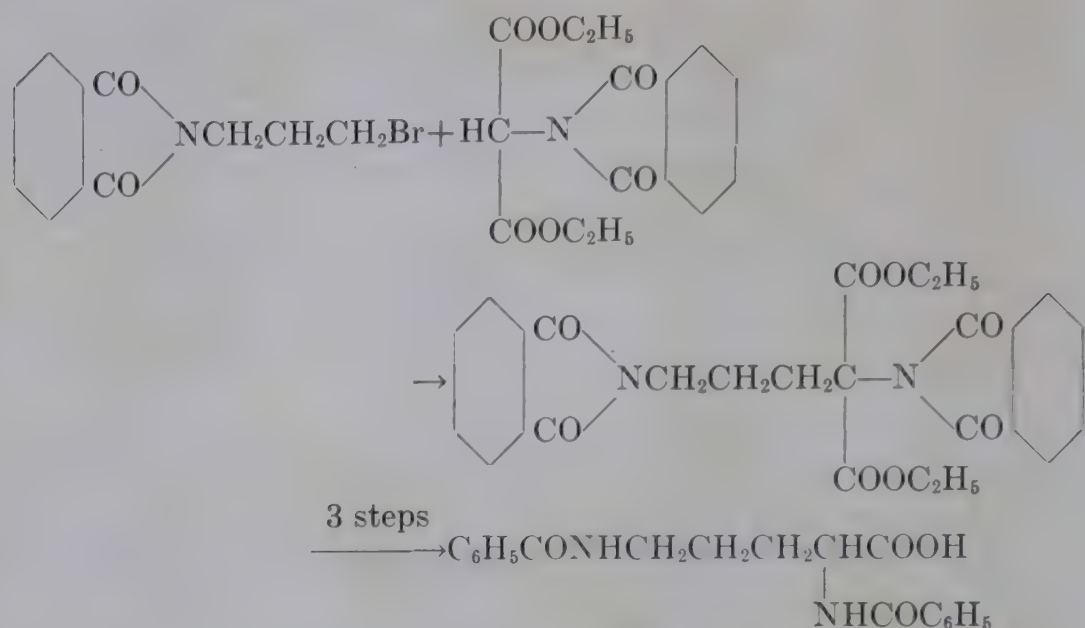


19. DL-Ornithine

Interest in this amino acid stems not only from the fact that it has been isolated from natural sources but also because of its close structural relationship to DL-arginine, DL-citrulline, DL-proline and DL-lysine. Fischer (107) synthesized dibenzoyl DL-ornithine, ornithuric acid, from ethyl malonate.



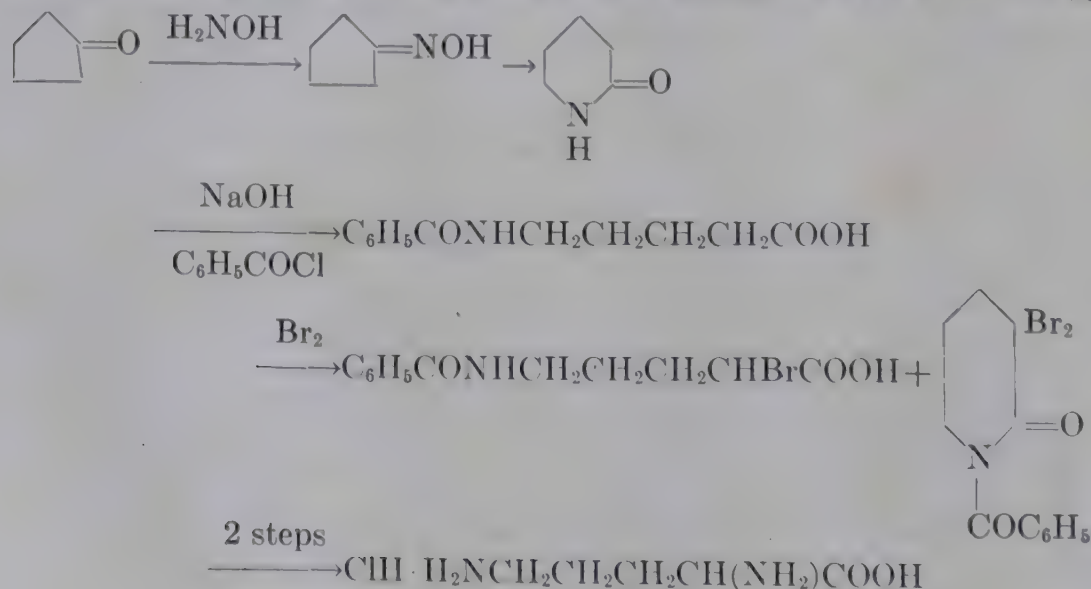
A very similar synthesis was carried out by Sørensen (245, 247) who used his ethyl phthalimidomalonate synthesis. The ester reacted with γ -bromopropylphthalimide to yield ethyl γ -phthalimidopropylphthalimidomalonate in 75% yield. Stepwise hydrolysis and decarboxylation completed the synthesis. Again, the dibasic amino acid was isolated as ornithuric acid.



Fischer and Zemplen (125) succeeded in preparing benzoyl DL-ornithine from piperidine in about 20% overall yield. The cyclic amine was benzoylated and then oxidized with potassium permanganate to δ -benzamidovaleric acid. This acid was brominated and aminated in the usual way to give δ -benzoylornithine.



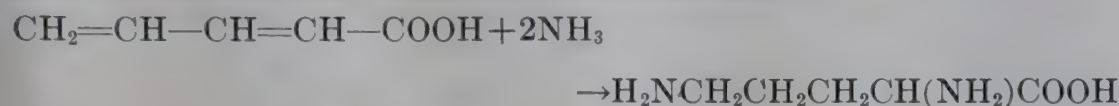
The intermediate, δ -benzamidovaleric acid was obtained in a different way by Fox, Dunn and Stoddard (129). They adapted the Eck and Marvel lysine synthesis to the preparation of the lower homolog. The overall yield in this synthesis was about 12%.



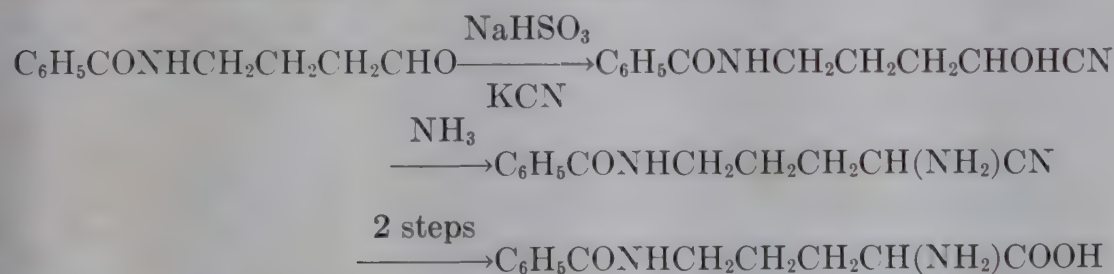
Maeda and Nozoe (184) also applied the Eck-Marvel synthesis to ornithine.

Adamson (6) found that hydrazoic acid reacted with α -amino-adipic acid to give DL-ornithine in 75% yield just as α -amino-pimelic acid gave DL-lysine.

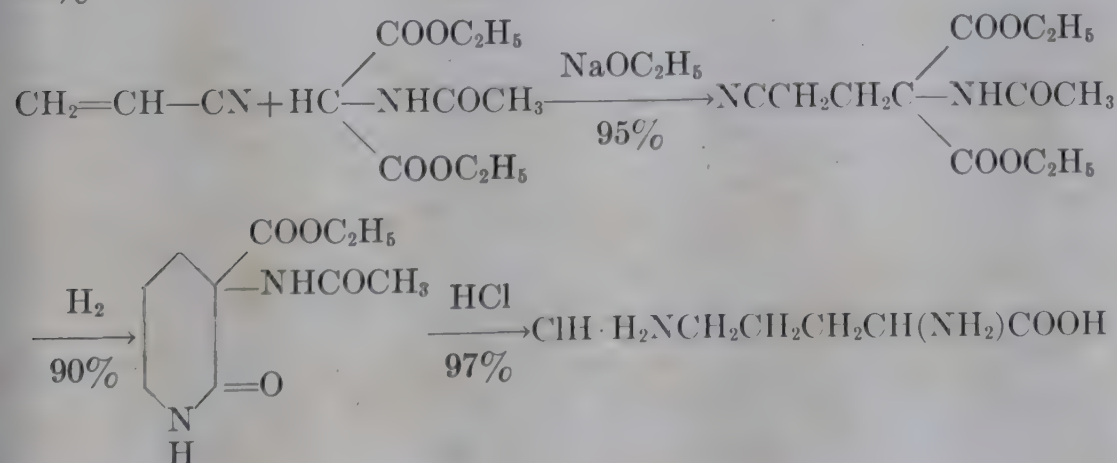
Fischer and Raske (119) succeeded in preparing DL-ornithine by the 1,4-addition of ammonia to vinylacrylic acid. Apparently the reaction is not a smooth one since a rather elaborate purification process was required to isolate the amino acid.



Keimatsu and Sugasawa (162) prepared benzoyl-DL-ornithine from γ -benzamidobutyraldehyde, an intermediate in their lysine synthesis, according to the following scheme.



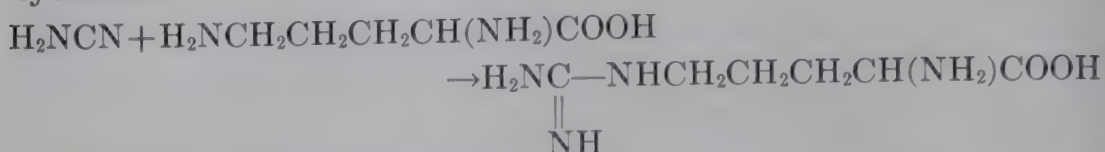
The above methods suffer the disadvantages of being either too long or of giving relatively poor overall yields. A convenient preparation of DL-ornithine was recently described by Albertson and Archer (10). The overall yield for the three steps shown below was 83%.



20. DL-Citrulline and DL-Arginine

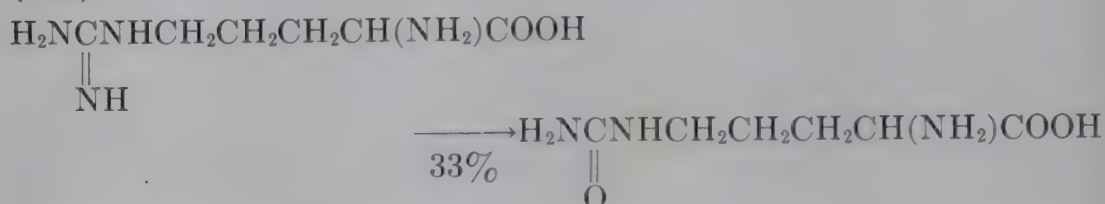
Schultze and Winterstein (236) prepared arginine from ornithine and cyanamide. A 37% conversion was realized when the reactants

were allowed to stand for three weeks in the presence of barium hydroxide.



Sörensen (248, 249) prepared benzoyl-DL-arginine from α -benzoyl-DL-ornithine. The latter substance was obtained in 52% yield by hydrolysis of ornithuric acid with barium hydroxide. When hydrochloric acid was used in the hydrolysis the main product was δ -benzoyl-DL-ornithine.

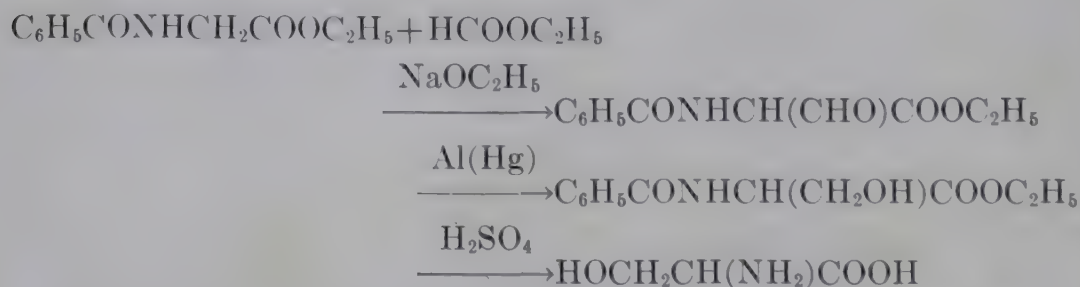
DL-Citrulline was prepared from arginine by alkaline hydrolysis (128).



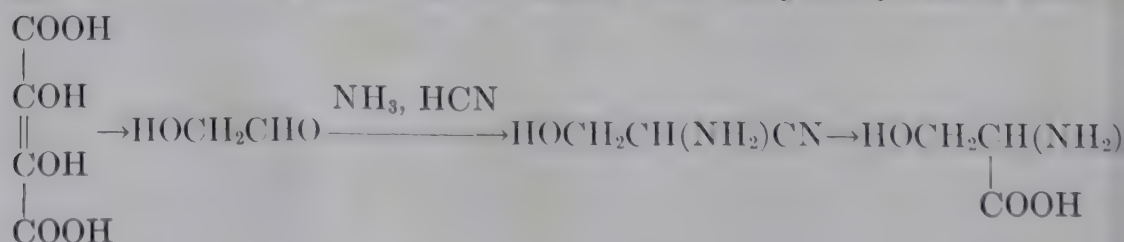
Kurtz (168) condensed the copper complex prepared from DL-ornithine and copper oxide with urea. The copper DL-citrullinate was decomposed to free DL-citrulline with hydrogen sulfide. The yield was 71% of the theoretical. The formation of the complex apparently protects the α -amino group. Fox (128) prepared this amino acid in a similar manner.

21. DL-Serine

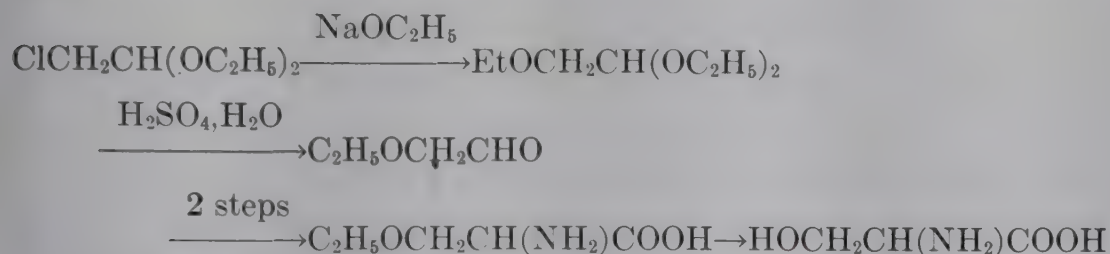
Erlenmeyer (88, 97) prepared DL-serine from ethyl hippurate and ethyl formate.



Fischer and Leuchs (116) were able to prepare DL-serine from glycollaldehyde which was obtained from dihydroxymaleic acid.



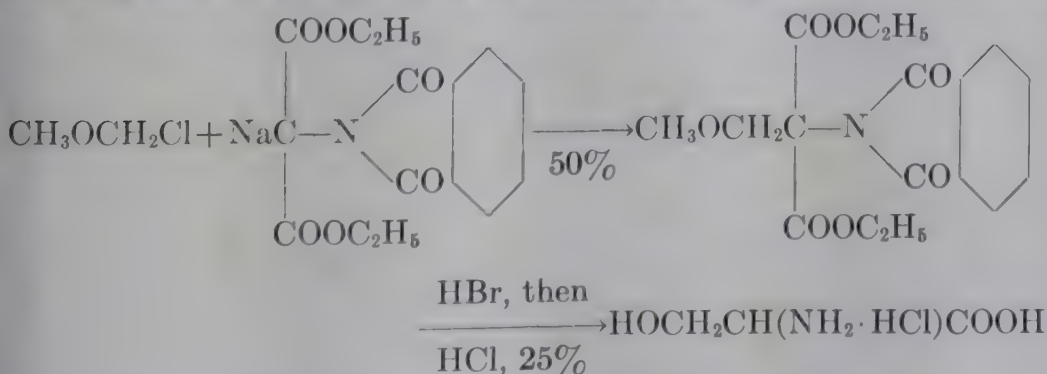
Leuchs and Geiger (174) carried out the reaction with the more accessible ethoxyacetaldehyde. The DL-serine-O-ethyl ether thus produced was de-ethylated with hydrobromic acid to give the hydroxy amino acid. Levene (171) found that better yields were obtained when bromoacetal was substituted for the chloro derivative.



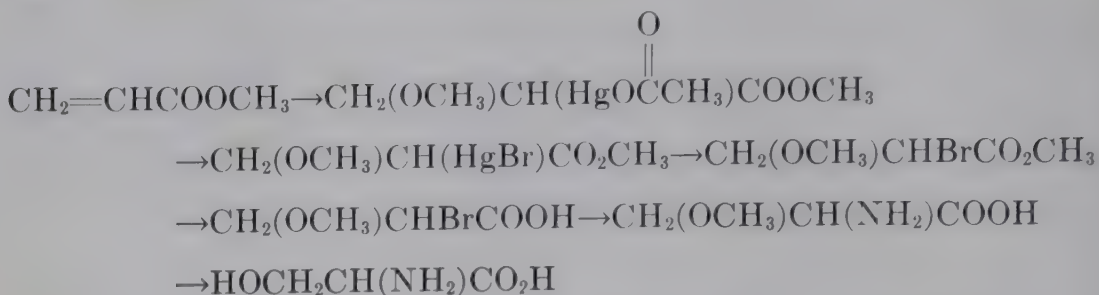
Dunn (67) showed that the Strecker reaction on ethoxyacetaldehyde followed by de-ethylation gave serine in 40% of the theoretical yield. The aldehyde was prepared by dehydrogenation of ethylene glycol monoethyl ether which is commercially available. The method has been improved recently by Redemann and Icke (220) who found that the yield in the dehydrogenation step may be increased to 35%. The last steps in the synthesis were carried through without isolation of the intermediates to yield the amino acid in about 45% based on the aldehyde.

A detailed study of the dehydrogenation of ethyl "Cellosolve" was carried out by Drake and coworkers (64) who were able to obtain the aldehyde in 43% of the theoretical yield.

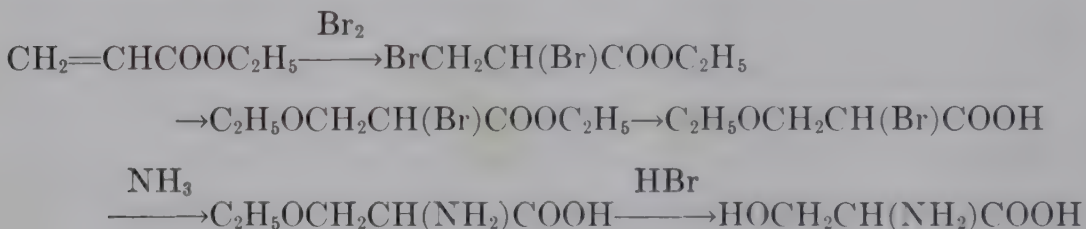
A synthesis from ethyl phthalimidomalonate has also been reported (192). Chloromethyl ether was condensed with the ester and the product was then subjected to the usual degradation reactions. By certain improvements in technique, Maeda, Terumi and Suzuki (185) managed to increase the yield in the first step to 83% and to 66% in the hydrolysis and cleavage reactions.



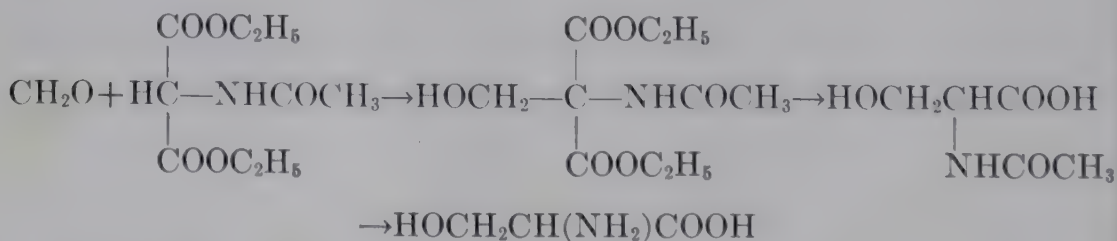
Schiltz and Carter (231) prepared DL-serine from methyl acrylate by means of a mercuriation reaction. The overall yield ranged from 30–40%.



An attractive synthesis of DL-serine from ethyl acrylate which avoided the use of mercury compounds was reported by Wood and du Vigneaud (274). Bromine was added to the unsaturated ester and without isolation the addition product was converted to ethyl α -bromo- β -ethoxypropionate by the action of sodium ethoxide. When subjected successively to saponification, amination and de-ethylation, serine was produced in 47% overall yield.

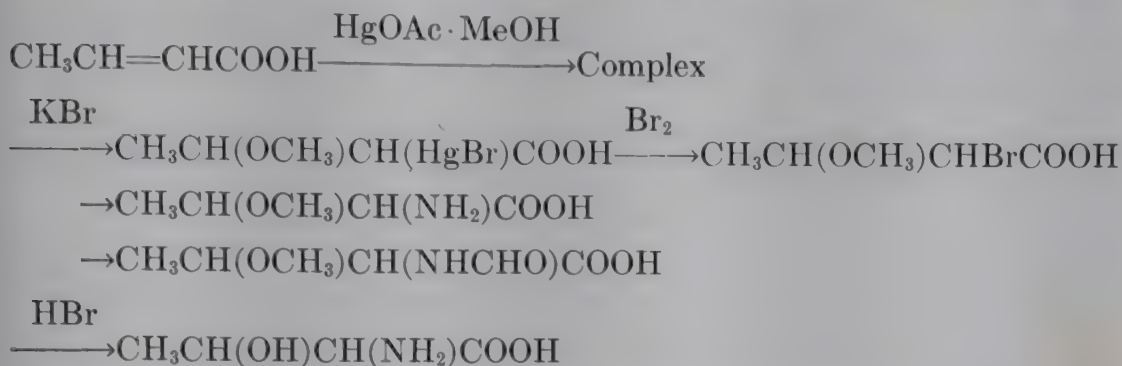


An elegant three stage serine synthesis was described by King (162a). Ethyl acetamidomalonate condensed quantitatively with formaldehyde to give the hydroxymethyl derivative which upon alkaline saponification and decarboxylation afforded acetyl DL-serine. The latter hydrolyzed to the amino acid. The overall yield was 65%.

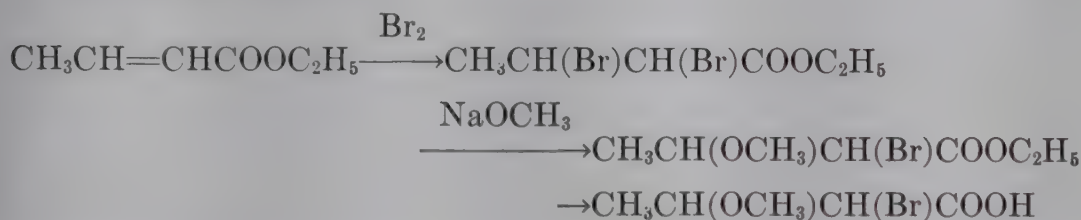


22. DL-Threonine

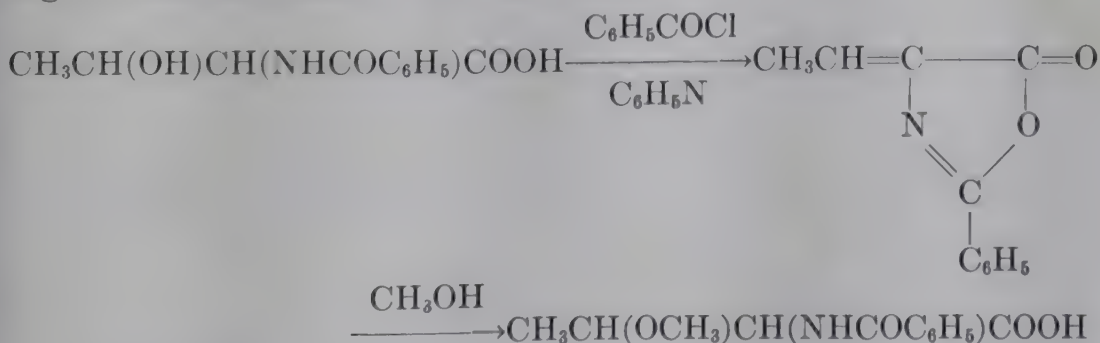
Mixtures of DL-threonine and the inactive isomer were prepared by Carter and coworkers from crotonic acid (43, 275) who followed the earlier work of Abderhalden (3). Later Carter (265) found that the isomers are best separated as the N-formyl-O-methyl derivatives. Pure DL-threonine was obtained by cleavage of the less soluble isomer. The overall synthesis thus became:



A simpler preparation of α -bromo- β -methoxybutyric acid was investigated by Carter and Ney (48) who proceeded according to the following equations:



Unfortunately the bromo acid yielded mainly the *allo* isomer. After amination and hydrolysis the addition of methylhypobromite to crotonic acid gave a bromo acid which also yielded only DL-*allo*-threonine after amination and demethylation (266). Since large amounts of biologically inactive DL-*allo*threonine accompany the natural isomer, methods were investigated for converting the former to the latter. Carter (46) reported that methanol added to benzoyl- α -aminocrotonic acid azlactone in cold benzene to give N-benzoyl-O-methyl-DL-threonine, a substance which was readily converted to the corresponding amino acid. Since the azlactone could be prepared from N-benzoyl-DL-*allo*threonine by the action of benzoyl chloride in pyridine a means was provided for transforming DL-*allo*threonine to DL-threonine.



The structure of the azlactone was proven by its synthesis (in poor yield) from acetaldehyde and hippuric acid. Mixtures of benzoyl-DL-threonine and benzoyl DL-*allo*threonine and mixtures

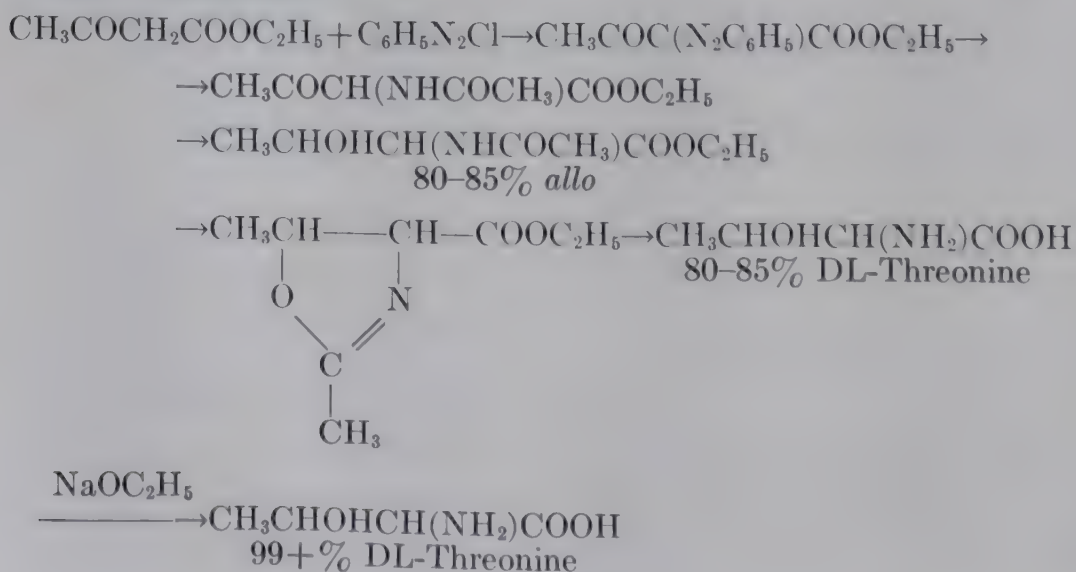
of the corresponding methyl ethers were separated as β -phenylethylamine salts (44).

The preparation of threonine from ethyl acetoacetate was investigated by Adkins and Reeve (7). The keto ester was nitrosated and the oximino keto-ester was reduced by two alternative routes. Direct reduction of ethyl oximinoacetoacetate was feasible only when carried out rapidly so that dihydropyrazine formation was avoided. The other procedure consisted of first, ethylating the oxime and then reducing the resultant O-ether. In either case, an ester was obtained which upon hydrolysis yielded a mixture of DL-threonine and DL-*allo*threonine. The purity of the preparation was not determined by biological means.

Birkhofer (27a) converted ethyl diazoacetoacetate to DL-threonine. No yields of pure amino acid were given.



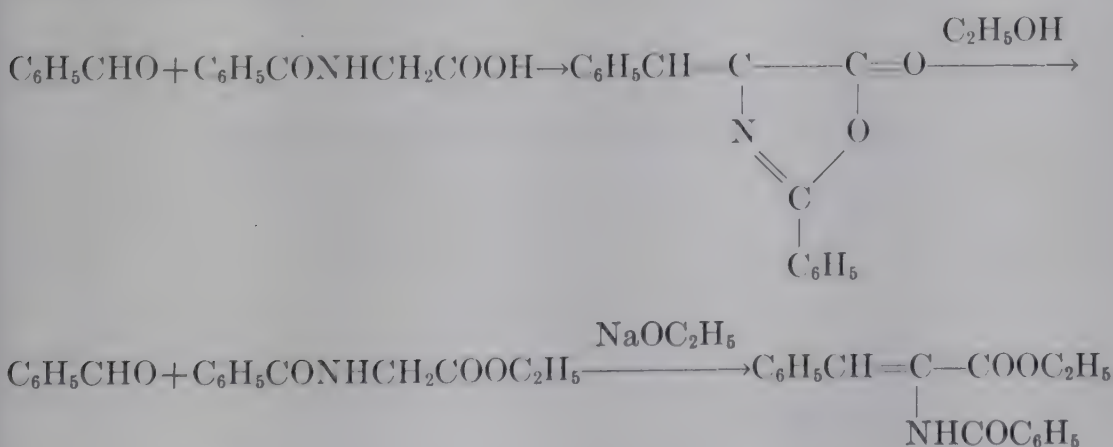
Tishler (211a) succeeded in preparing pure DL-threonine from ethyl acetoacetate. The keto-ester condensed with benzenediazonium chloride to give the phenylazo derivative which was reductively acetylated to ethyl α -acetamido-acetoacetate. The latter on further reduction gave a mixture of ethyl esters of N-acetyl DL-*allo*threonine and N-acetyl-DL-threonine. The former comprised about 85% of the mixture. When treated with thionyl chloride a mixture of oxazolines was produced which was hydrolyzed to DL-*allo*threonine and DL-threonine. As a result of the last two steps a Walden inversion occurred so that the final mixture now consisted largely of the desired isomer. Separation of the two was effected through the sodium salt in ethanol solution. DL-Threonine was prepared in 57% overall yield.



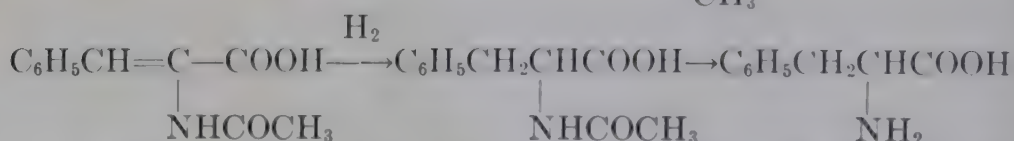
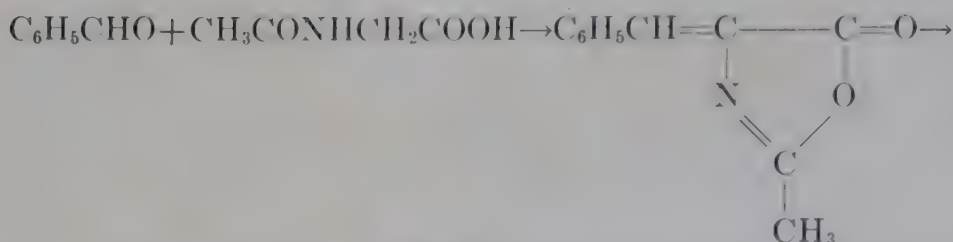
Attenburrow (16a) prepared DL-threonine by almost the same procedure. The product from the interaction of acetic anhydride and hippuric acid was treated with ethanol. The ethyl α -benzamido-acetoacetate thus formed was reduced and converted to the mixture of oxazolines. These were fractionally crystallized and the proper isomer hydrolyzed to DL-threonine. Some time later Elliott (76a) reported that when the mixed oxazoline esters were hydrolyzed to the corresponding acids, the derivative in the *allo* series underwent a Walden inversion whereas the other compound did not. Thus the mixture of esters on careful saponification gave only one oxazoline acid thus obviating the necessity for a separation. When this modification was used the overall yield of DL-threonine was 60%.

23. DL-Phenylalanine

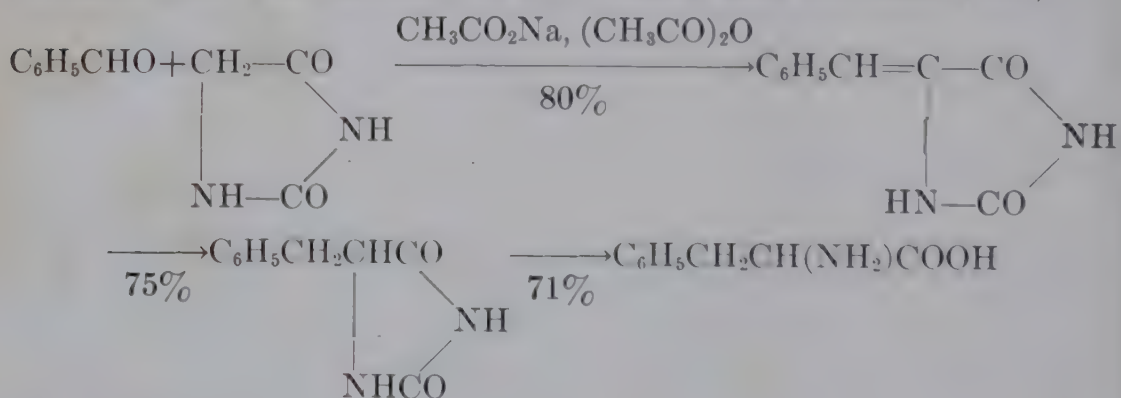
This amino acid has been prepared by almost all the known methods for preparing amino acids. Plöchl (213) condensed benzaldehyde with hippuric acid, hydrolyzed the intermediate to α -benzamidocinnamic acid and then reduced the double bond with sodium amalgam. In a series of papers extending over a period of about twenty years Erlenmeyer (79, 80, 84, 85) clarified the structures of the intermediates and demonstrated the usefulness of this method of amino acid synthesis. He showed that the initial condensation product was an azlactone (81) and proved that the ethanolysis product was ethyl- α -benzamidocinnamate by independent synthesis (83).



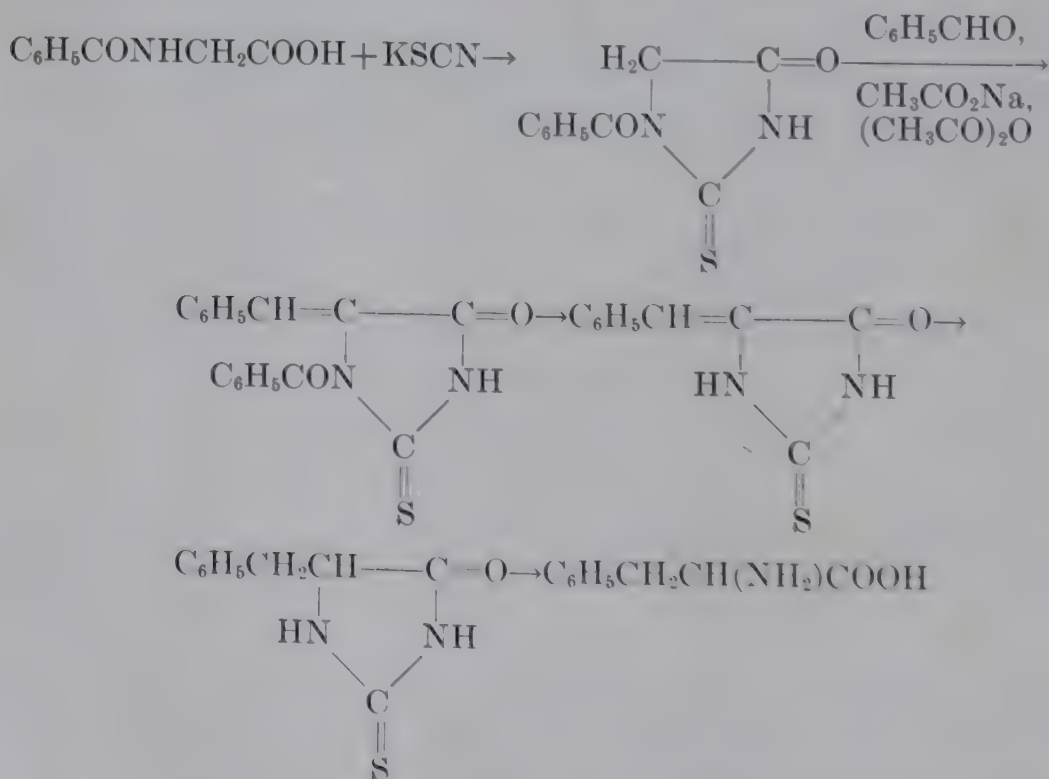
The synthesis of DL-phenylalanine from aceturic acid and benzaldehyde was carried out by Erlenmeyer (82) who obtained the azlactone, and by Herbst and Shemin (148) who performed the reduction and hydrolysis.



The preparation of DL-phenylalanine from hydantoin and benzaldehyde was carried out by Wheeler and Hoffman (267).

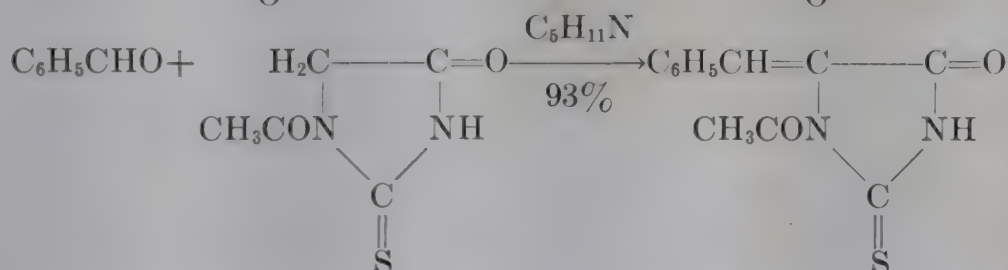
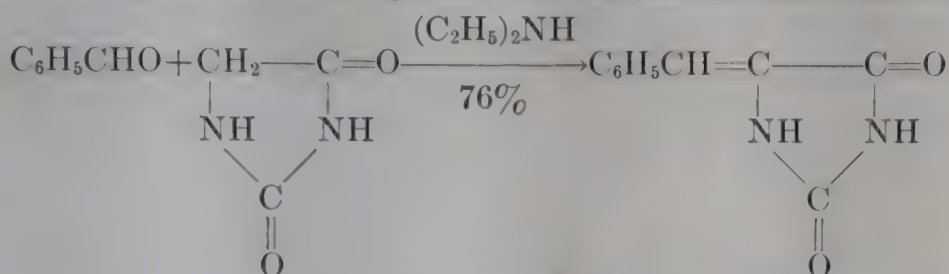


Thiohydantoins may be used also to prepare DL-phenylalanine (155).

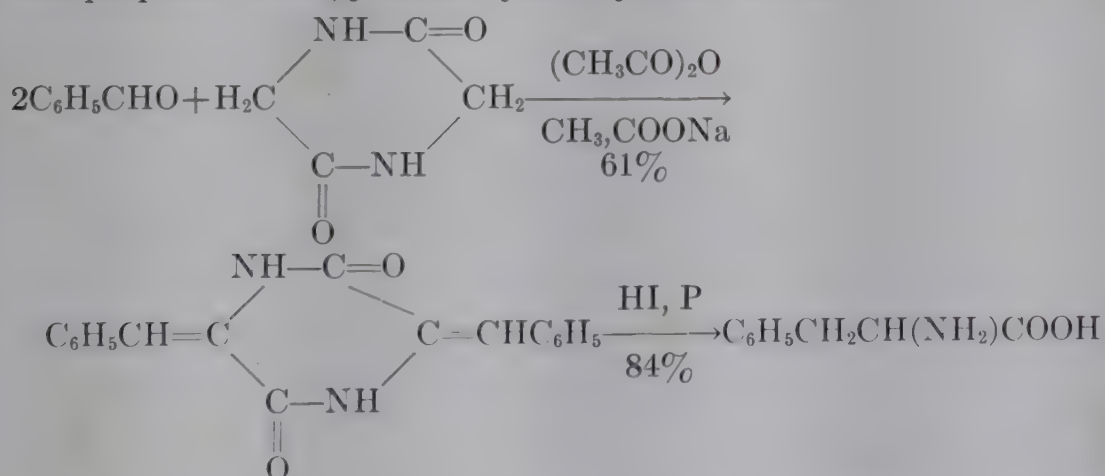


Boyd and Robson (32) found that the condensation of hydantoins and thiohydantoins with aldehydes took place in the presence of

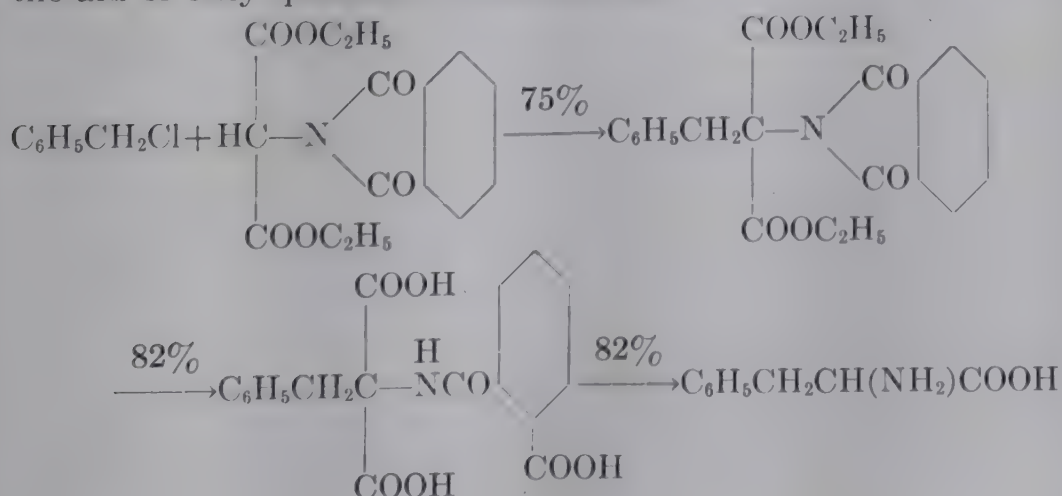
basic catalysts. Excellent yields were obtained in several instances.



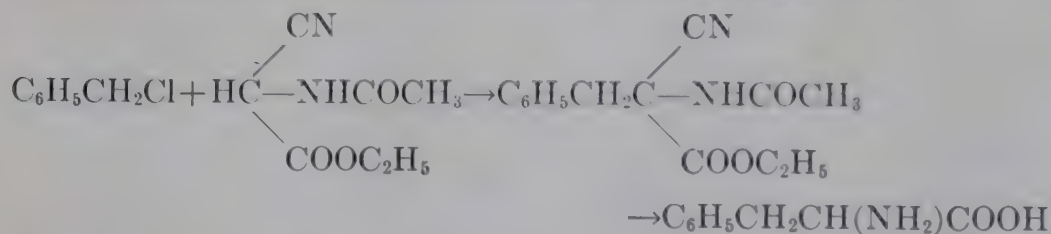
Sasaki (227) found that glycine anhydride condensed with aldehydes to form 3,6-diarylidene-2,5-diketopiperazines which were reduced and hydrolyzed to the amino acids. DL-Phenylalanine was prepared in 50% overall yield by this method.



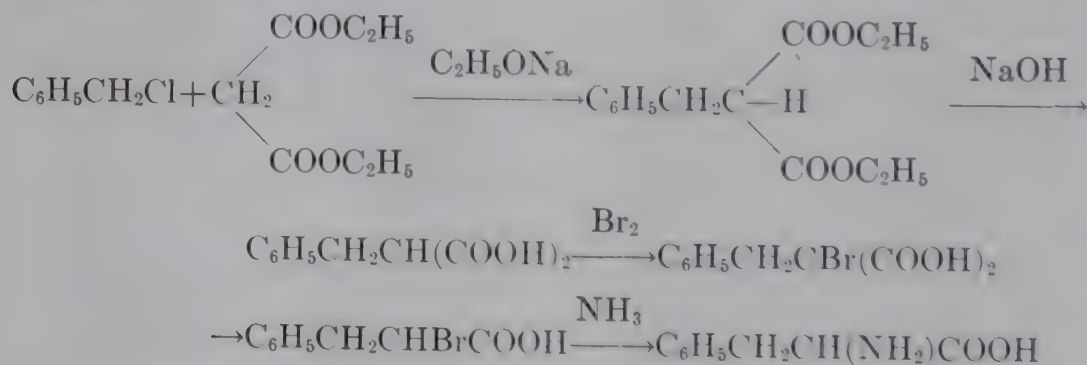
DL-Phenylalanine has been prepared by Sørensen (245) with the aid of ethyl phthalimidomalonate ester.



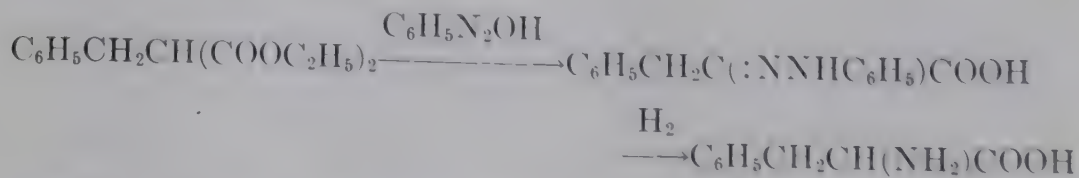
Dunn (69) used ethyl benzamidomalonate in a similar synthesis to obtain the desired amino acid in good yield. Both ethyl acetamidomalonate (9) and ethyl acetamidocyanoacetate have been used to prepare DL-phenylalanine. The preparations with the cyano ester afforded the amino acid in 61% overall yield (14).



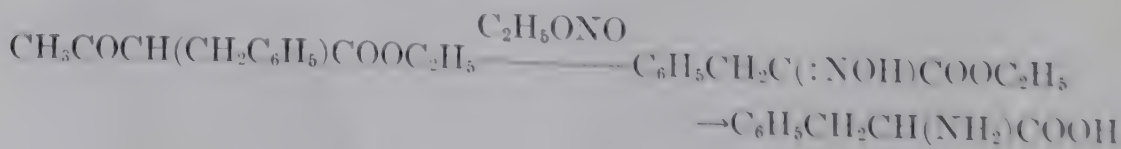
The preparation from α -bromohydrocinnamic acid has been reported by Fischer (109) and Marvel (189). The required intermediate was prepared by a malonic ester synthesis. The overall yield was about 35%.



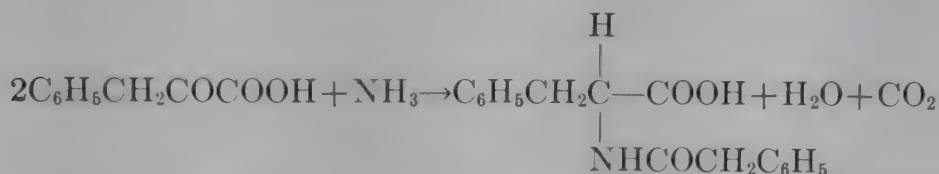
A synthesis from ethyl benzylmalonate was discovered by Feofilaktov (103). When coupled with benzenediazonium chloride and then hydrolyzed the ester gave the phenylhydrazone of phenylpyruvic acid in 60% yield. The latter was reduced by conventional means to DL-phenylalanine.



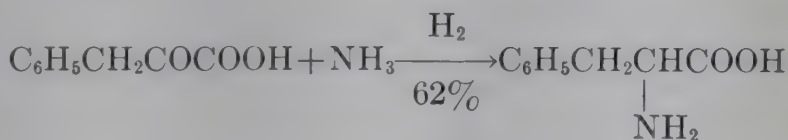
Two preparations from ethyl benzylacetoacetate are known (100, 103). In one of these the keto ester was treated with benzenediazonium chloride and the resulting hydrazone reduced and hydrolyzed to DL-phenylalanine. Hamlin and Hartung (139) reduced the corresponding oxime which was prepared by a similar method. Excellent yields were reported.



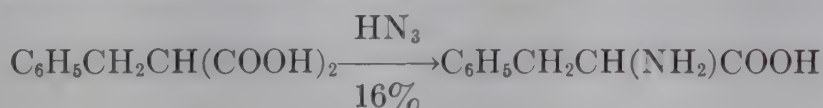
Apparently Erlenmeyer (92) was the first to prepare DL-phenylalanine from phenylpyruvic acid. When the keto acid was treated with ammonia, phenacetylphenylalanine resulted. On hydrolysis the amino acid was generated.



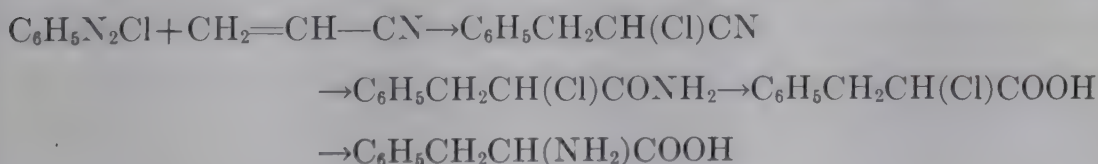
Reduction of phenylpyruvic acid by catalytic means was mentioned by Knoop (164).



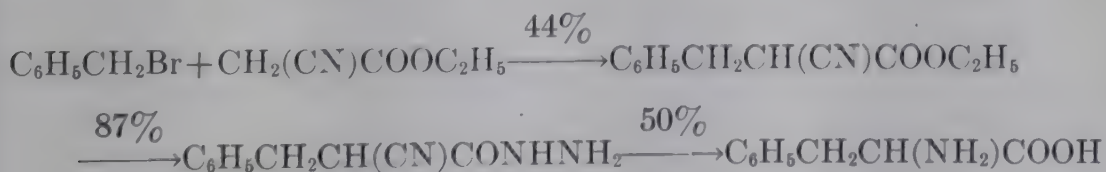
DL-phenylalanine has been prepared from phenylacetaldehyde by a Strecker reaction (96). A Schmidt reaction with benzylmalonic acid gave phenylalanine in 16% yield (36).



Gaudry (134, 135) prepared phenylalanine in 17% overall yield by an interesting series of reactions starting with acrylonitrile.

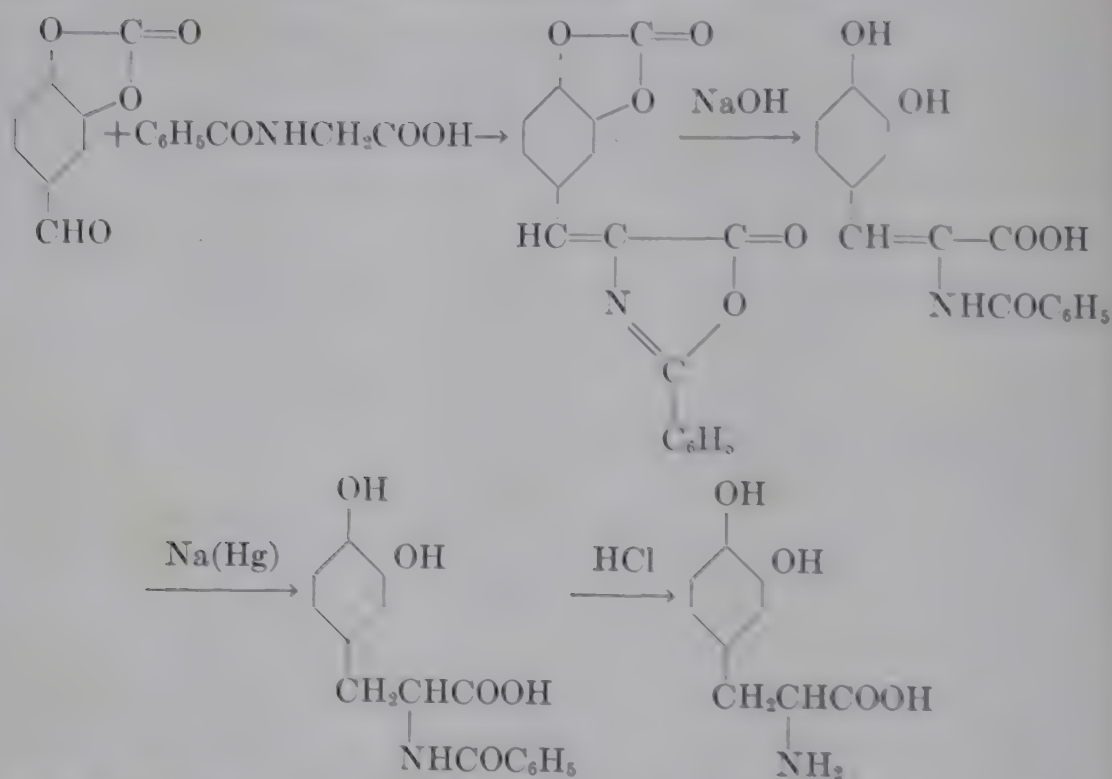


Gagnon, Gaudry and King (132) used the Darapsky method to synthesize DL-phenylalanine.



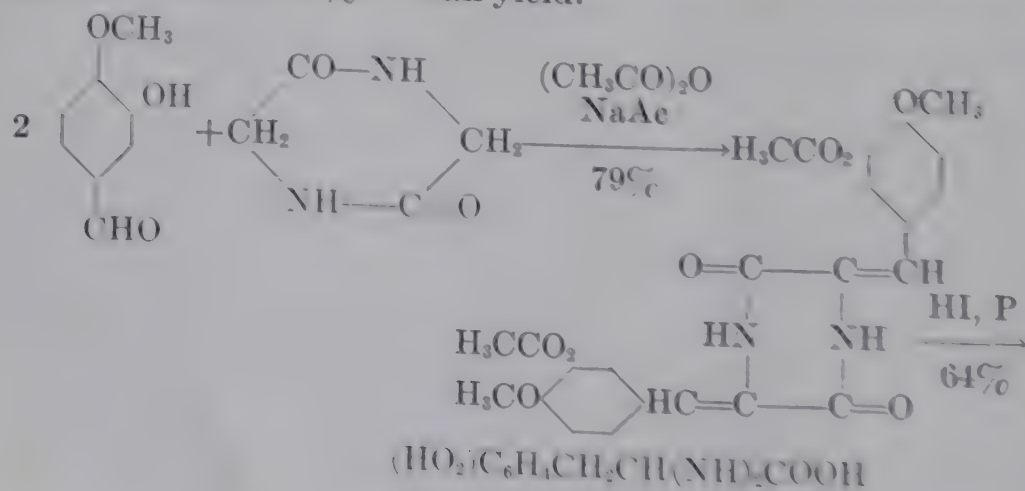
24. DL-Dihydroxyphenylalanine

Funk (131) prepared this amino acid by an Erlenmeyer-Plöchl synthesis. The hydroxy groups in protocatechuic aldehyde were first protected as the carbonate. The azlactone which resulted was hydrolyzed and reduced to benzoyl dihydroxyphenylalanine, which was then hydrolyzed with hydrochloric acid to the free amino acid.



Sugii (254) claimed that he was unable to cleave the azlactone which was prepared from protocatechuic aldehyde and hippuric acid. However, when vanillin replaced the more sensitive un-methylated aldehyde, no difficulties were encountered. The demethylation and debenzoylation steps were performed simultaneously by heating with dilute hydrochloric acid in sealed tubes at elevated temperatures.

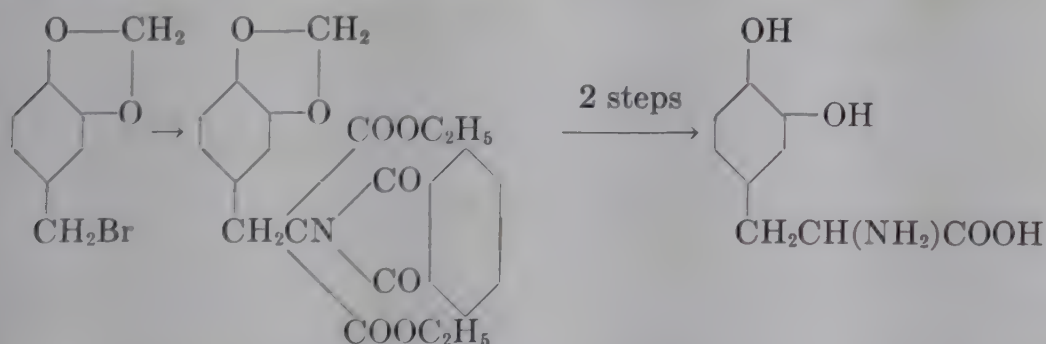
Hirai (151) condensed vanillin with glycine anhydride (2,5-diketopiperazine) in the presence of acetic anhydride and sodium acetate to give the acetylated azlactone which upon treatment with hydriodic acid and phosphorus suffered reduction, deacetylation and demethylation at the same time; the desired amino acid was obtained in 55% overall yield.



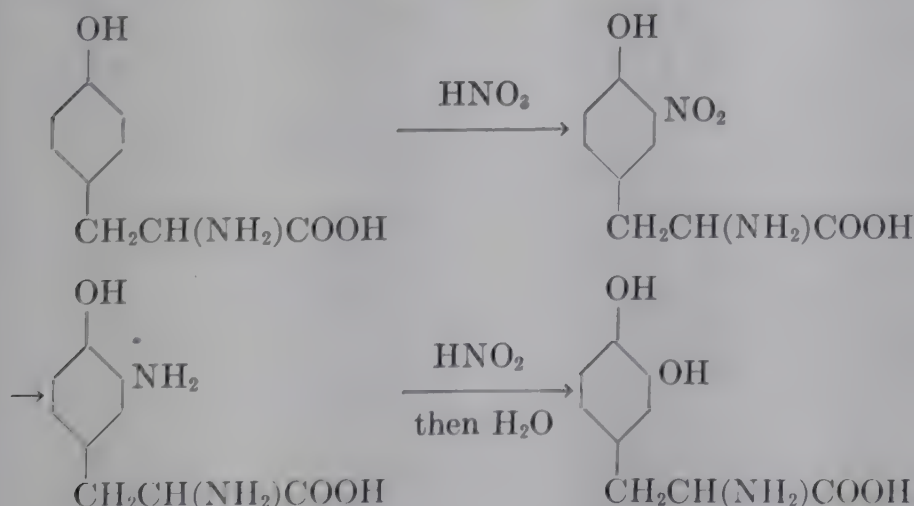
Harington and McCartney (143) agreed that the hydriodic acid-reduced phosphorus reduction represented a considerable advance over the older sodium amalgam method.

Harington (146) started with protocatechuic aldehyde and acetic acid in another synthesis of DL-dihydroxyphenylalanine. The azlactone resulting therefrom was hydrolyzed to the acrylic acid which was reduced catalytically to β -(3,4-diacetoxyphenyl)- α -acetamidopropionic acid. Resolution was accomplished with brucine. The active amino acids were obtained after deacetylation with hydrochloric acid.

Stephen and Weizmann (250) applied the Sørensen synthesis to the preparation of this amino acid.



An enzymatic synthesis from tyrosine was recorded by Raper (218). He succeeded in isolating dihydroxyphenylalanine as a product of the action of tyrosinase on L-tyrosine. Waser and Lewandowski (263) succeeded in carrying out this transformation chemically. L-Tyrosine was nitrated and the resulting nitro body reduced with tin and hydrochloric acid. The diazonium salt was prepared in sulfuric acid and then hydrolyzed by addition to boiling aqueous cupric sulfate. The L-form of the amino acid was eventually obtained.



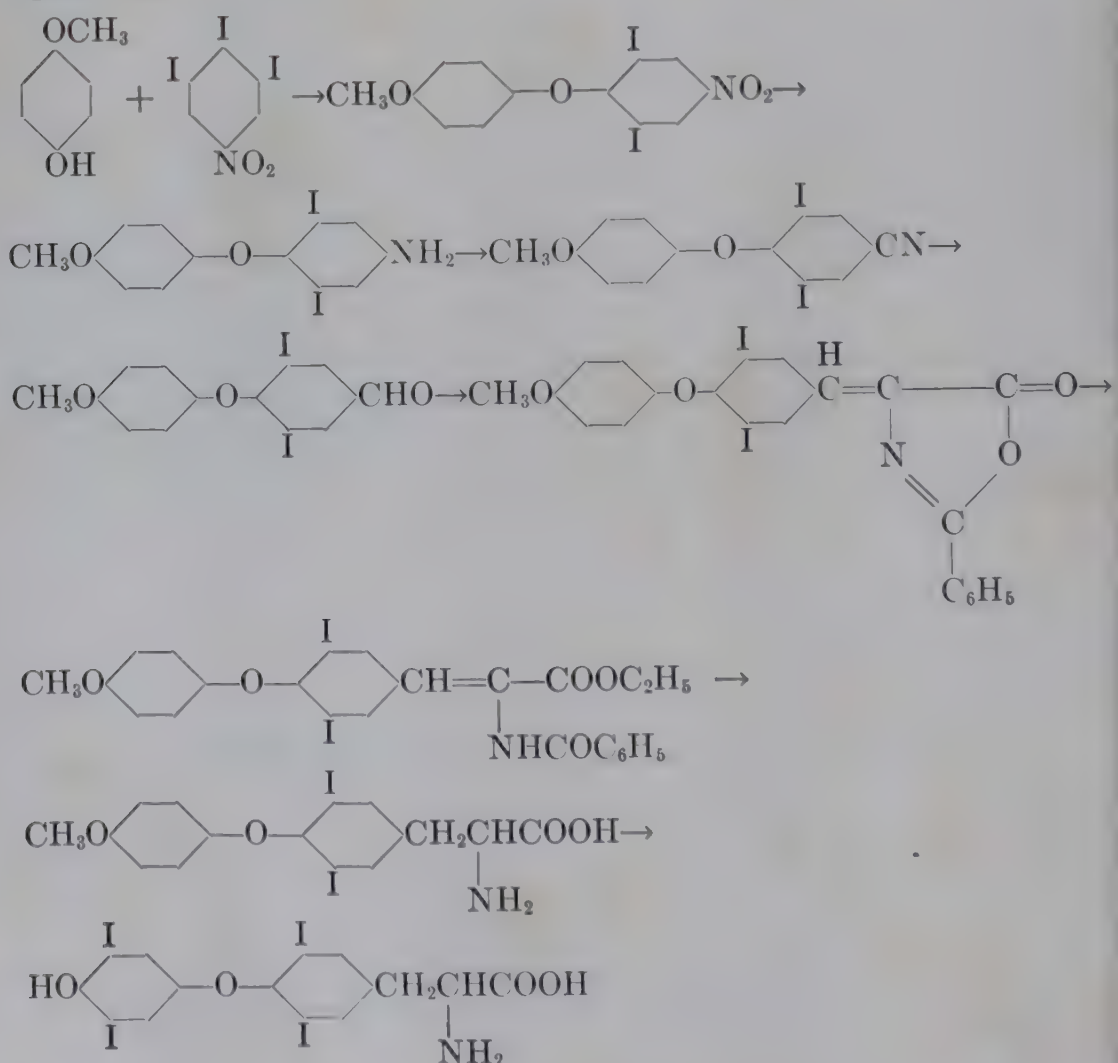
25. DL-Diiiodotyrosine

All preparations of this amino acid start from L-tyrosine. The iodination was carried out in alkaline solution by Henze (147) and Wheeler and Jamieson (268). Oswald (207) found that by lowering the temperature of the reaction the yield and purity of the product could be much improved in the Wheeler method. Savitskii (228) obtained the amino acid in 88% yield by iodinating a sodium hydroxide solution of tyrosine with a solution of iodine in potassium iodide.

Recently Block and Powell (28) obtained a pure sample of DL-diiiodotyrosine by the action of iodine monochloride on L-tyrosine in acetic acid.

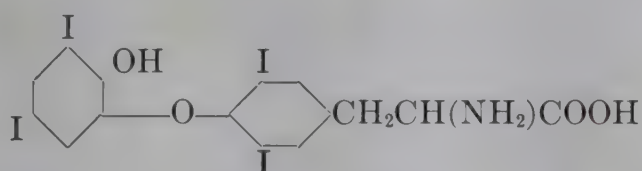
26. DL-Thyroxine

Harington culminated his work on the active principle of the thyroid gland by effecting a synthesis and resolution of the substance, DL-thyroxine. The synthesis is illustrated by the following equations.

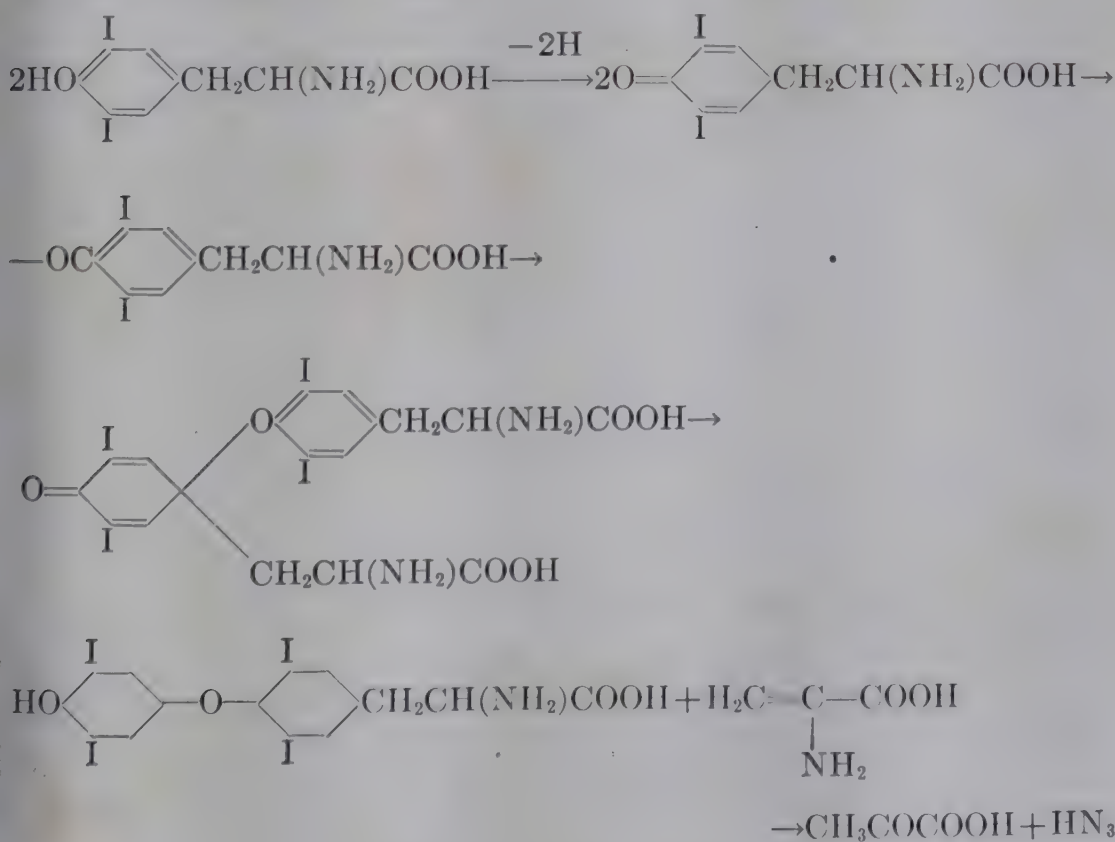


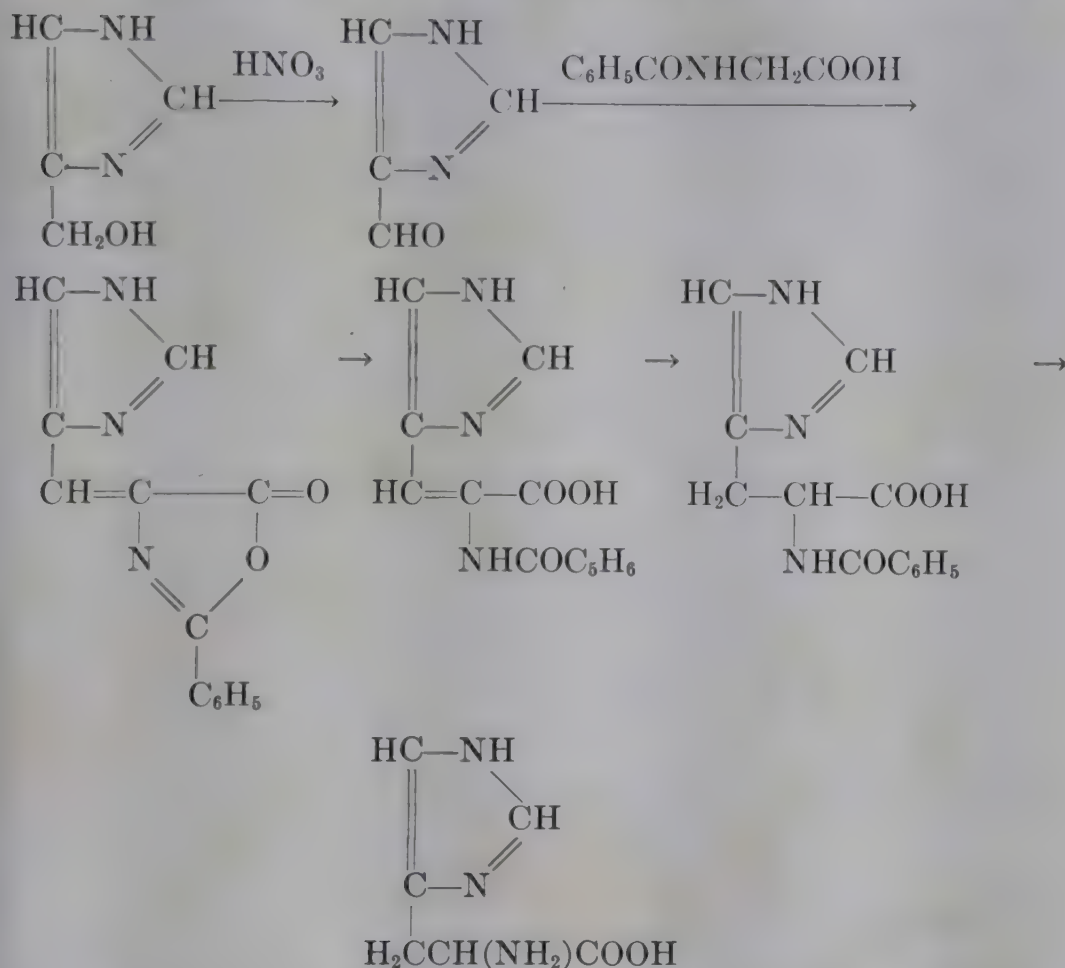
It was found later (143) that the conversion of the azlactone to the cinnamic ester could be eliminated entirely by reducing directly with hydriodic acid and phosphorus. Savitskii (229) claimed to have improved the yields in this synthesis. However, the same steps were used as in the Harington method.

The racemic amino acid was resolved by fractional crystallization of the L-phenylethylamine salt of DL-formylthyroxine. The L-form was three times as active physiologically as the enantiomorph (141). An isomer of thyroxine, DL-3,5-diiodo-4-(3,5-diiodo-2-hydroxyphenoxy)phenylalanine also possesses thyroxine-like activity (194).

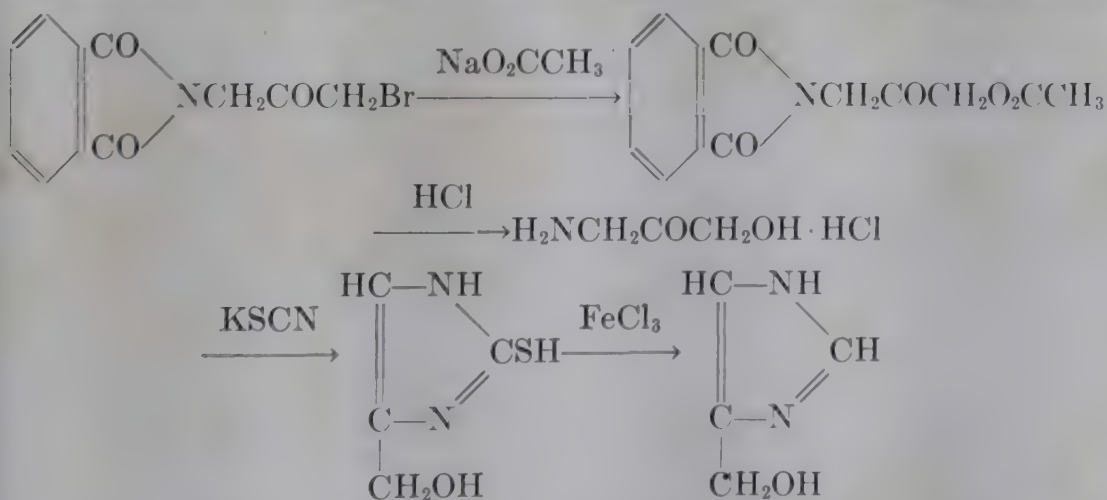


An interesting preparation of thyroxine was discovered by von Mutzanbecker (183, 193) and confirmed by others. When diiodotyrosine was kept at 37° in a slightly alkaline medium small yields of thyroxine were obtained. DL-Thyroxine was also found among the hydrolytic products of iodinated casein (183). Johnson and Tewkesbury (158) proposed the following mechanism for the oxidation of diiodotyrosine.



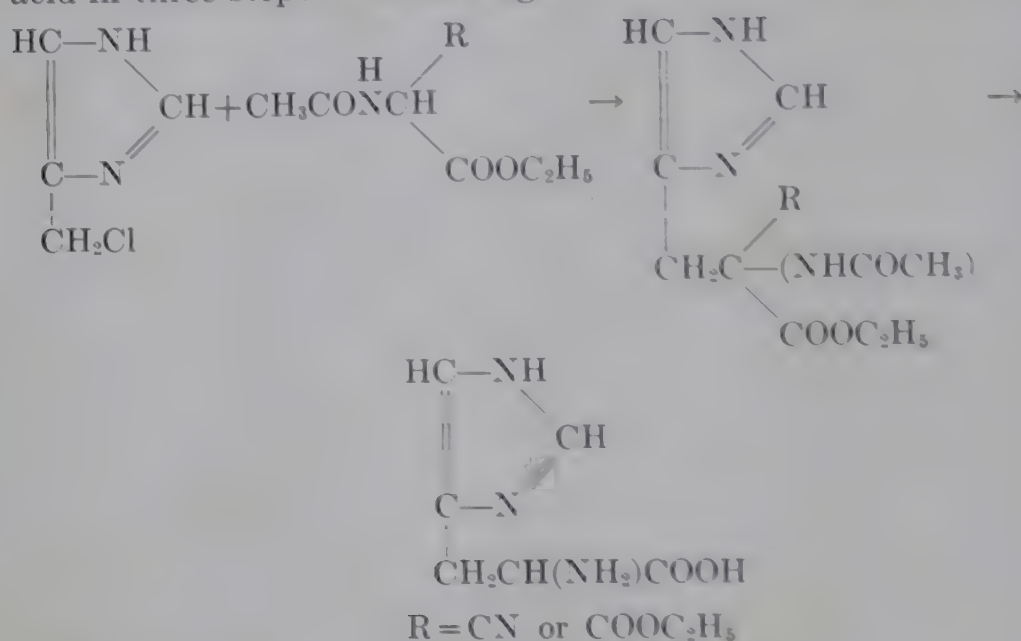


The intermediate hydroxymethylimidazole was prepared by Jackson and Marvel (154) according to the following scheme.



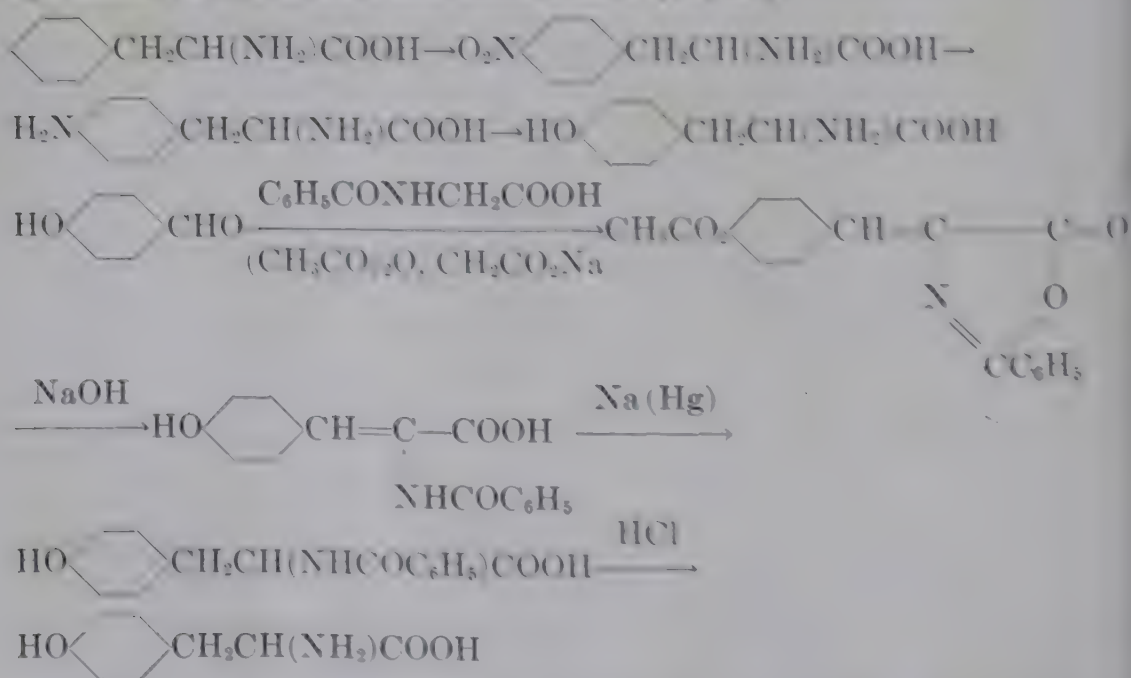
A more convenient synthesis of this substance was reported by Darby, Lewis and Totter (58) who found that oxidation of fructose in the presence of formaldehyde and basic cupric carbonate in ammonia solution gave the desired product. Albertson and Archer (9) and later Albertson and Tullar (14) employed this reaction in

their preparation of DL-histidine. The hydroxymethylimidazole was converted to chloromethylimidazole and the latter then condensed with either ethylacetamidomalonate or ethylacetamidocynoacetate. Hydrolytic degradation of the esters gave the amino acid in three steps from the sugar.



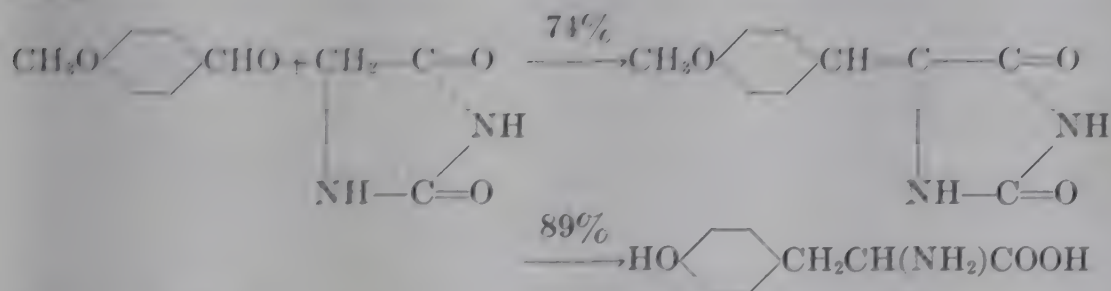
28. DL-Tyrosine

Erlenmeyer (94, 95) converted DL-phenylalanine to DL-tyrosine by a three stage synthesis. He also employed the azlactone synthesis to synthesize this amino acid (90, 91).



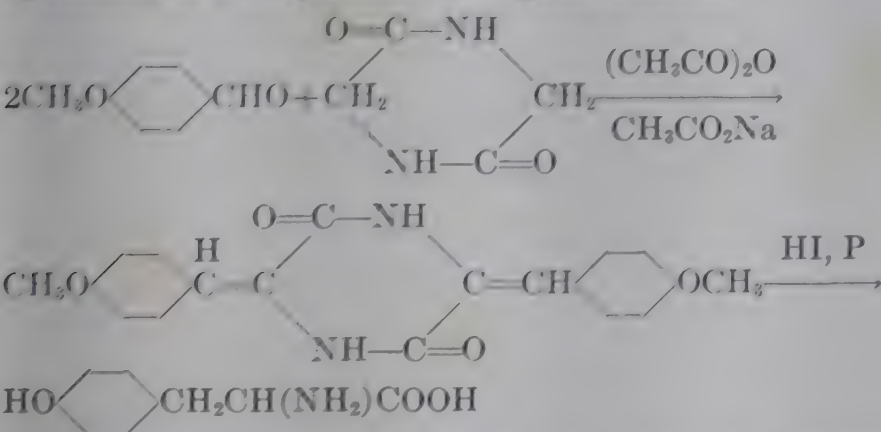
Fischer (106) improved the reduction step from 10-15% yield to 70%.

The preparation from hydantoin was carried out by Wheeler and Hoffman (267) and Johnson and Hahn (156). The thiohydantoin synthesis was applied by Johnson and Nicolet (157) to this amino acid.

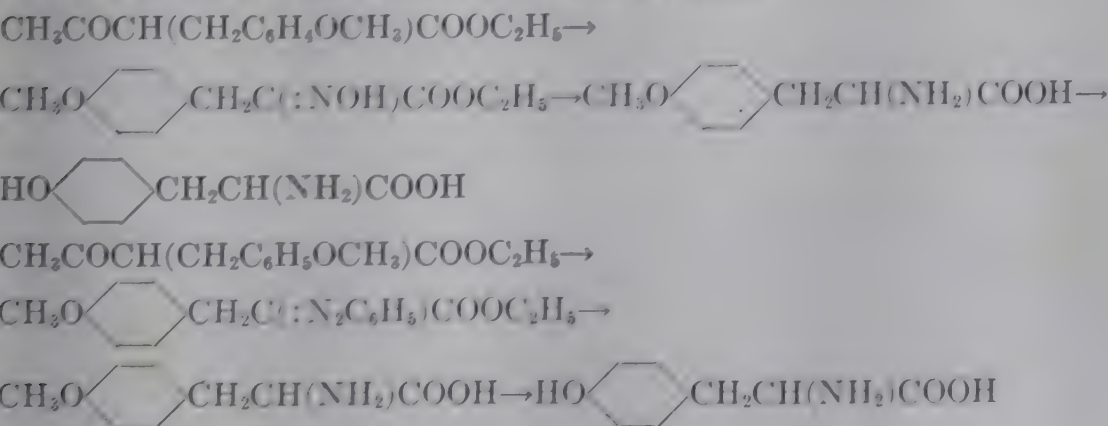


Boyd and Robson (32) investigated the reaction of aromatic aldehydes and hydantoins in the presence of basic catalysts. Good yields were realized when either anisaldehyde or *p*-hydroxybenzaldehyde were condensed with hydantoin in the presence of ethylamine. Acetylthiohydantoin gave excellent yields with a piperidine catalyst.

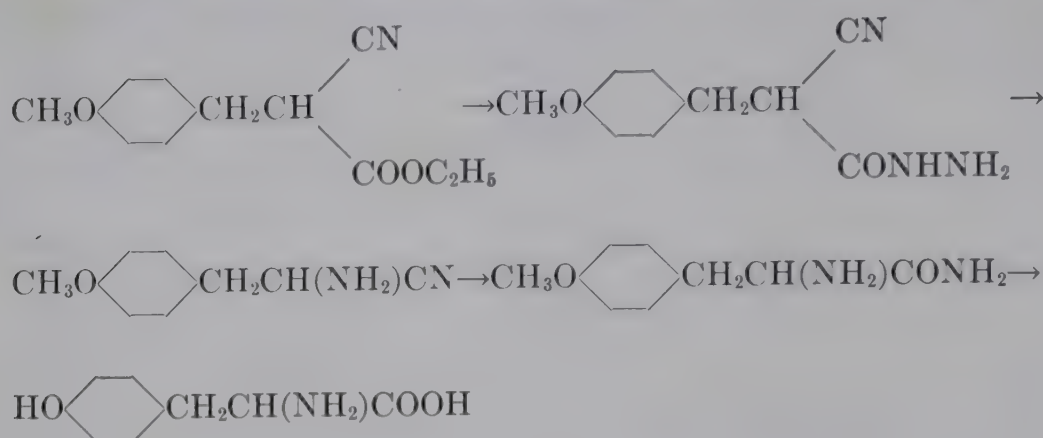
Sasaki (227) showed that glycine anhydride may be used in the synthesis of DL-tyrosine with good results.



Various alkylation methods have been used to synthesize tyrosine. Hamlin and Hartung (139) and Feofilaktov (105) have applied their methods to the synthesis of DL-tyrosine.

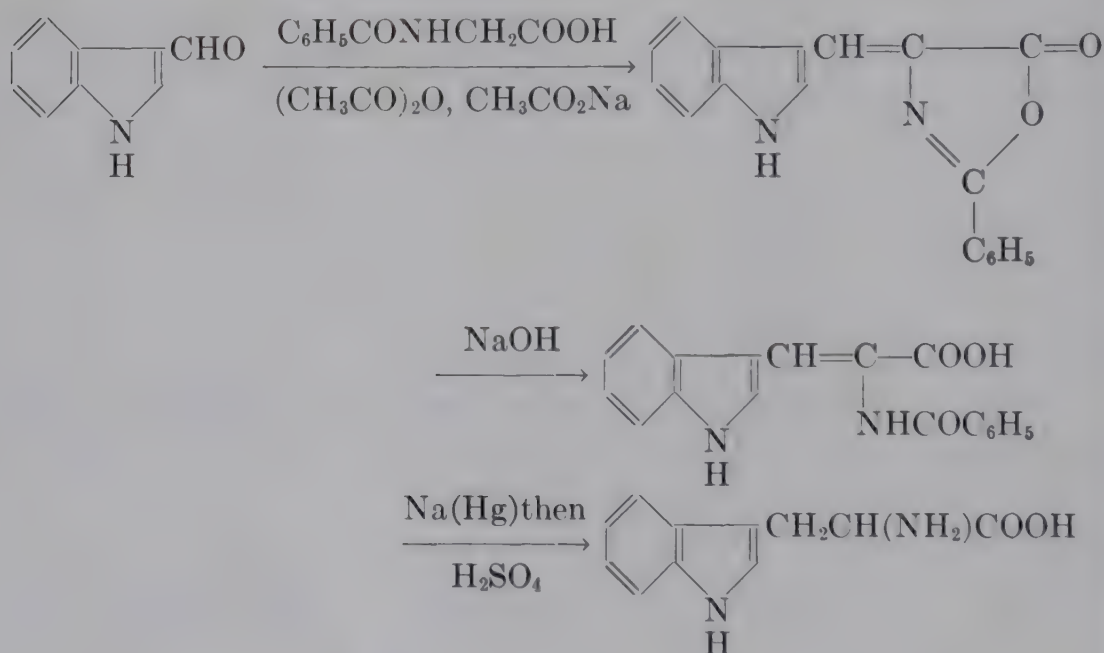


Gagnon, Gaudry and King (132) used the Darapsky synthesis.

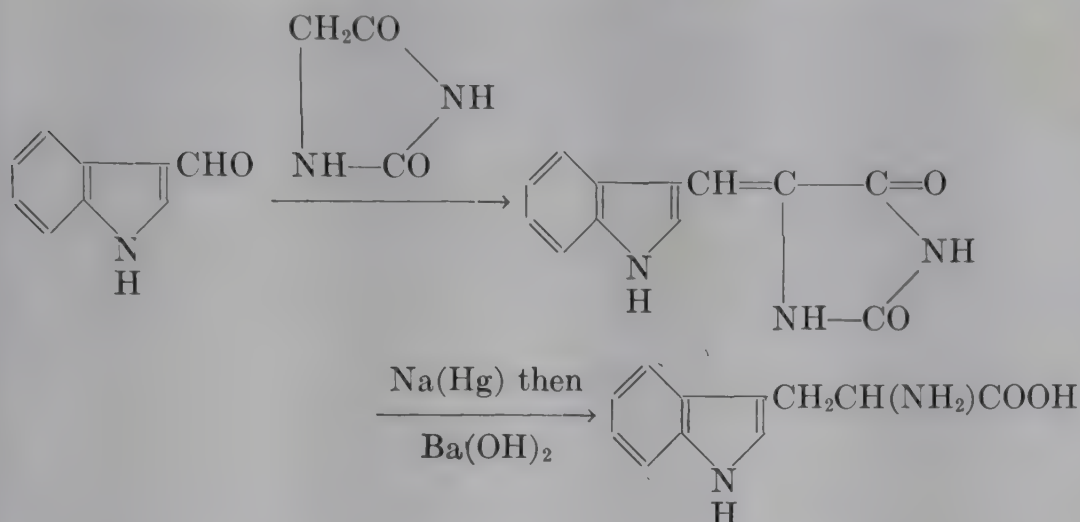


29. DL-Tryptophan

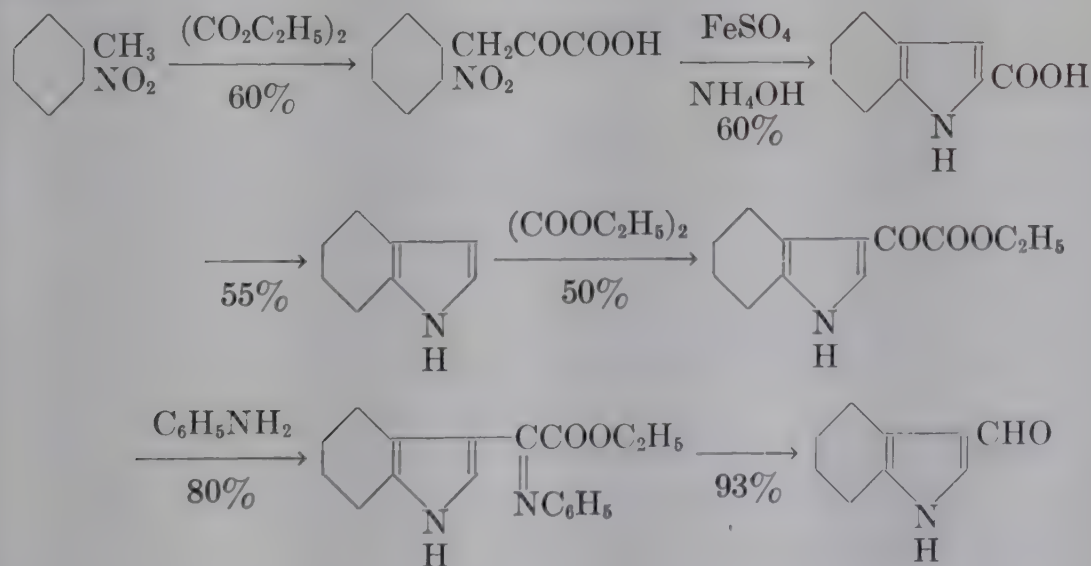
Until recently, DL-tryptophan was synthesized exclusively from indole-3-aldehyde. Ellinger and Flamand (76) and Restelli (221) used the Erlenmeyer-Plöchl synthesis. Majima (186) used the hydantoin synthesis. He prepared the requisite aldehyde from indole-magnesium bromide and ethyl formate.



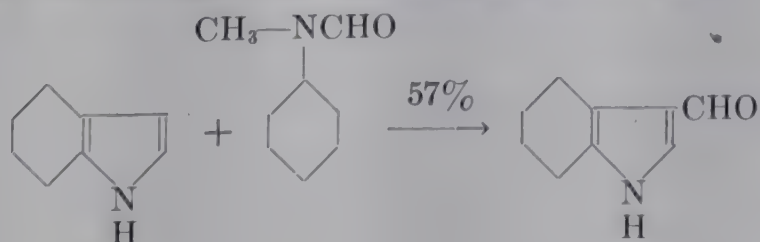
Boyd and Robson (33) used a similar method to prepare this amino acid. The aldehyde was condensed with hydantoin in the presence of piperidine in 65% yield. Reduction with ammonium sulfide for 500 hours gave the amino acid in 70% yield.



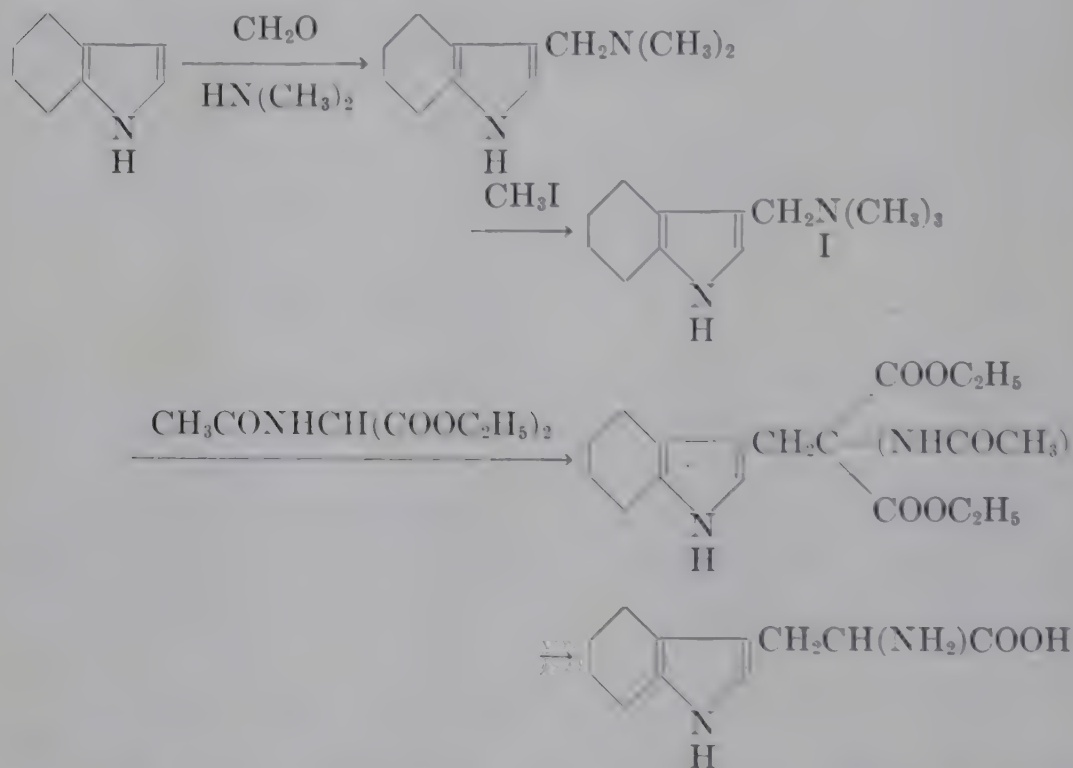
The chief drawback in the above methods was the preparation of the aldehyde. Attention was directed toward improving the yield in the preparation of this compound. A synthesis from *o*-nitrotoluene was recently described (75). The Boyd-Robson synthesis was then followed, with considerable increase in overall yield. The β -indolemethylhydantoin was reduced catalytically in quantitative yield.



Recently it was found that indole-3-aldehyde may be prepared conveniently according to the following equation (237).



A modified Sørensen synthesis was employed by Snyder (244) and Albertson (12) and their coworkers. These authors discovered that gramine methiodide could be used as an alkylating agent. The reactions from indole are:



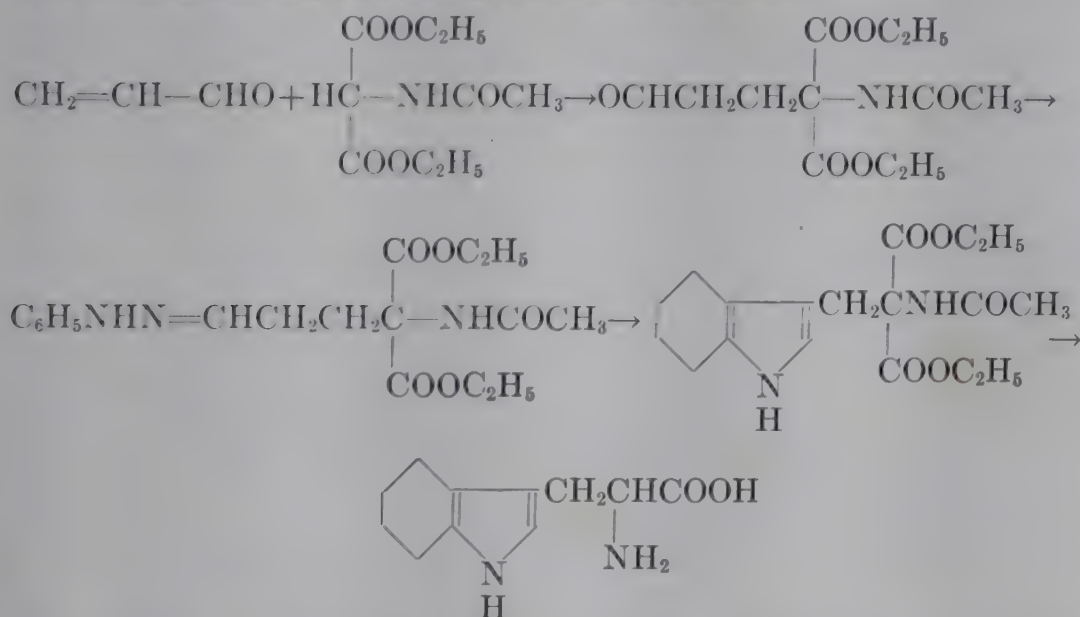
Albertson and coworkers (13) found that if two moles of methyl sulfate is added to a mixture of one mole of gramine and one mole of ethylacetamidomalonate in alcoholic sodium ethoxide the yield in the alkylation step is 95%. When gramine was heated with ethyl acetamidomalonate in boiling xylene with powdered sodium hydroxide as a catalyst, alkylation occurred to the extent of 90% (152).

Tryptophan was prepared in 71% overall yield from indole when ethyl acetamidocyanoacetate was substituted for the corresponding malonate (14).

A variant of the above method was explored by Little and Weisblat (179a). They used ethyl nitroacetate in place of ethyl acetamidomalonate. The ester condensed with gramine to give ethyl α -nitro- β -(3-indolyl)-propionate which was reduced and hydrolyzed to the amino acid. The overall yield from gramine was 50%.

An interesting DL-tryptophan synthesis which did not use indole as the starting material was reported recently (192a, 262b). Acrolein was condensed with ethyl acetamidomalonate and the resulting aldehydo-ester isolated as the phenylhydrazone. The

latter was subjected to a Fischer indole synthesis where upon the same ester resulted as obtained from gramine and ethyl acetamidomalonate. After hydrolysis and decarboxylation the amino acid was secured in 45% overall yield. The steps are:



A feature of this synthesis not shared by others is its adaptability to the preparation of nuclear substituted derivatives of DL-tryptophane.

B. THE PREPARATION OF POLYPEPTIDES

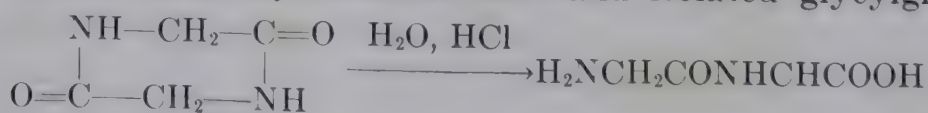
The hydrolytic cleavage of protein molecules with acids, bases or enzymes eventually results in a mixture of α -amino acids. How these amino acids are linked together has been a problem which faced protein chemists for many years. It is generally believed that the α -amino and α -carboxylic acid groups play a major part in the combinations which are present in proteins. Further, it is known that the enzymatic cleavage of protein molecules results in the liberation of equal numbers of amino and carboxyl groups. A linkage which would satisfy this requirement is the peptide bond, $-\text{CONH}-$, which on treatment with water would liberate a free carboxyl and free amino group. Powerful tools which would give information about the structure of proteins and also the chemistry of enzymes are synthetic polypeptides. Most of the progress in this field may be attributed to Emil Fischer and his student, Max Bergmann. The latter's carbobenzoxy synthesis is the most versatile and elegant method yet devised for the preparation of peptides. Table I illustrates the types of peptides which have been synthesized by different methods.

TABLE I
SYNTHETIC PEPTIDES

Method of Synthesis	Peptides Synthesized	References
Hydrolysis of diketopiperazines	Glycylglycine, glycylleucine, leucylglycine, serylserine, histidylhistidine	1
	Cysteylcysteine	2
Ester condensation	Lysyllysine	1
Azlactone	Phenylalanylarginine, tyrosylarginine	3
Hydrochlorides of amino acid chlorides	Leucylglycine, leucyldiglycine, phenylalanylglycine	1
Halogen acylhalide	A large number of peptides of glycine, alanine, cystine, leucine, phenylalanine, aspartic acid, and proline, containing as many as 18 amino acids. Examples: alanyldiglycine, leucylpentaglycylglycine	1
Carbobenzoxy method	A large number of peptides of glycine, alanine, proline, cystine, serine, tyrosine, glutamic acid, aspartic acid, lysine, and histidine. Examples: glycylproline, lysylhistidine, glutamylglutamic acid, etc.	4
	Carnosine (β -alanylhistidine) and related histidine peptides	5
	Glutathione (γ -glutamylcysteylglycine)	6, 7
	Asparthione (β -aspartylcysteylglycine)	8
	Cysteyl- and cystyltyrosine, and tyrosylcysteine and -cystine	9
	Cystinyldiglycine, cystinyldidiglycine, and diglycylcystine	10, 11
	Serylserine, serylglycine, serylalanine, serylglutamic acid	12
	Prolylcysteyltyrosine	13

1. Diketopiperazine synthesis

Fischer and Fourneau (113) hydrolyzed 2,5-diketopiperazine with concentrated hydrochloric acid and isolated glycylglycine.



When a mixed anhydride, such as glycylleucine anhydride was hydrolyzed a mixture of glycylleucine and leucylglycine resulted (123). This method of preparation has its severe limitations. Only dipeptides can be prepared, mixtures invariably result when mixed

anhydrides are used, the ease of hydrolysis varies for each substance so that new sets of conditions must be developed for each preparation and the threat of considerable racemization of optically active diketopiperazines is always present.

2. Polymerization of amino acid esters and anhydrides

Fischer (110) found that peptide esters lost alcohol on heating and form higher peptides. This type of reaction has been extended by Frankel (130) and Pacsu (208) to the preparation of polypeptides of the simpler amino acids. Frankel prepared polypeptides of glycine containing 12 to 20 units by heating ethyl glycinate. Methyl glycinate gave polymers containing 18 to 30 units. Further heating resulted in a decrease in alkoxyl content corresponding to polymers containing 42 units from the ethyl ester and 110 from the methyl ester. Similarly ethyl alaninate gave initially substances which analyzed for polymers containing 10 to 16 amino acid units. At 150° the 14 unit polymer condensed further to yield compounds containing 17 to 23 units.

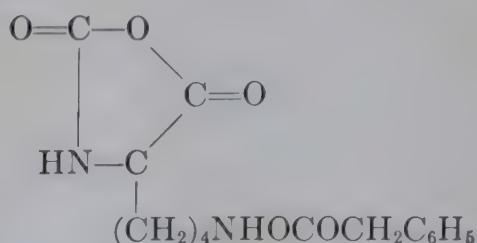
The polymers showed positive biuret tests and hydrolyzed to the parent amino acids in high yields.

Katchalski and coworkers (158a) found that a polycarbobenzyloxyllysine could be prepared which contained 32 amino acid units.

REFERENCES TO TABLE I

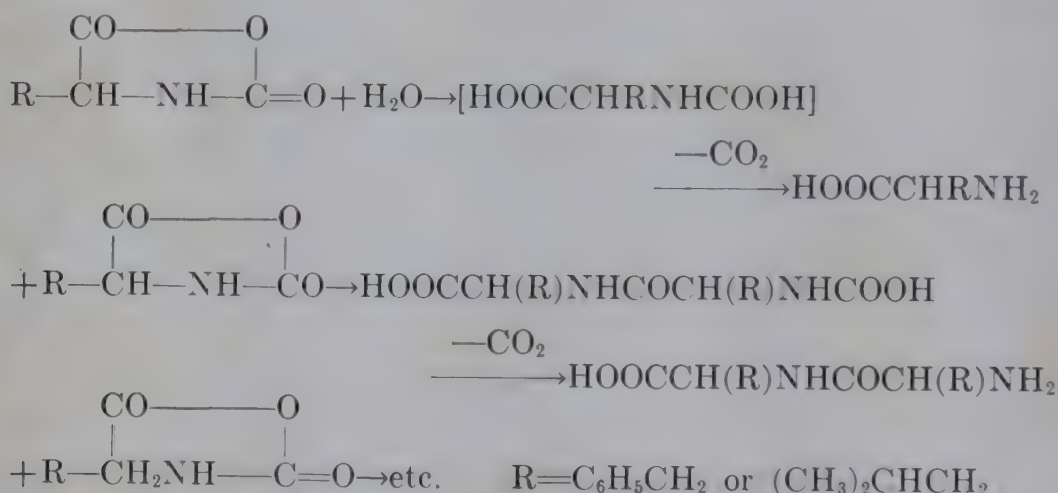
1. FISCHER, E.: Untersuchungen über Aminosäuren, Polypeptide Proteine, Springer, Berlin, 1906.
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The anhydride



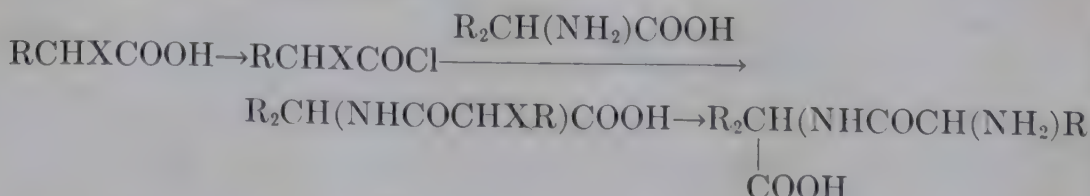
suffered loss of carbon dioxide on heating and underwent self-condensation.

Woodward and Schramm (275a) used similar derivatives of phenylalanine and leucine and succeeded in preparing polymers the molecular weight of which varied from one to fifteen million. A trace of water served to initiate the polymerization which occurred in benzene solution. The reaction was formulated in the following way:



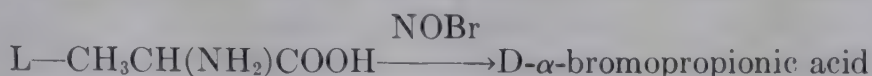
3. Amination of haloacylamino acids

A further step toward solving the problem of peptide synthesis was taken by Fischer (108, 117, 118) when he found that the haloacyl derivatives of amino acids, on amination, yielded peptides. The halo acid was converted to the corresponding acid chloride and the latter coupled with an amino acid. Amination of the resulting amide gave the dipeptide.

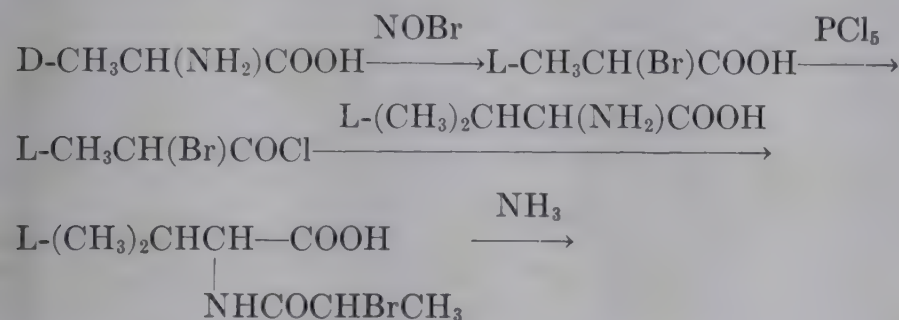


The process could be repeated with another molecule of haloacyl chloride. In this way Fischer (111) was able to prepare peptides containing up to 18 amino acid units. This method was applicable

to the preparation of optically active peptides. Since Walden inversion occurred in the following reaction, Fischer (121) started



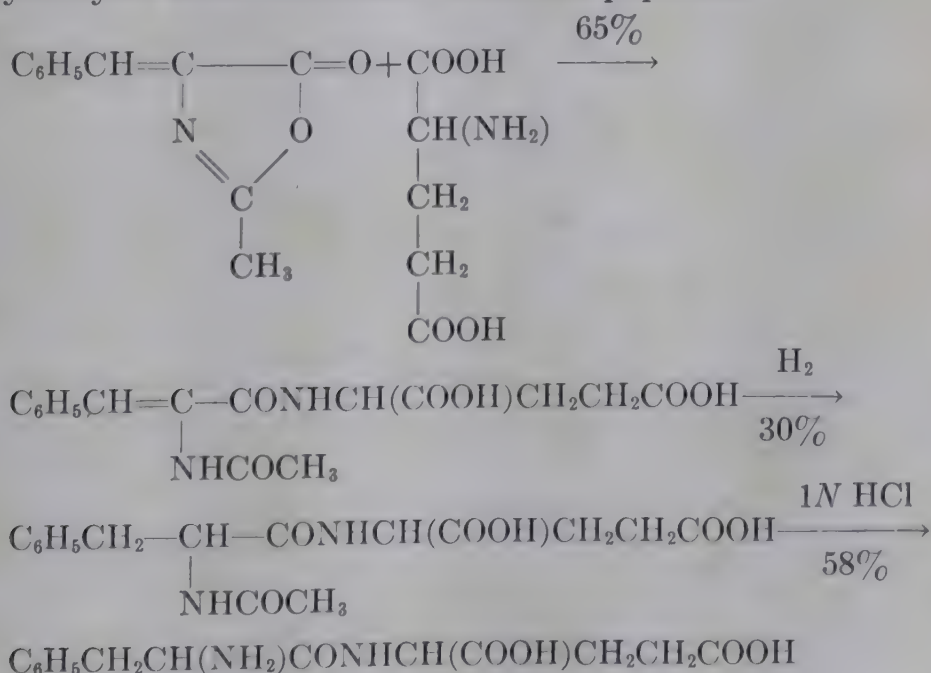
with the unnatural isomer which he obtained through resolution of the racemic acid. L-Alanyl-L-valine was prepared according to the following reaction.



Despite the fact that this method was an obvious improvement on previous procedures, certain difficulties still remained. The more complex amino acids could not be used, partial racemization occurred with the optically active compounds and considerable manipulative difficulties were encountered in the preparation of some of the higher peptides.

4. Azlactone synthesis

Bergmann (23) found that certain azlactones reacted with amino acids to give unsaturated acylated peptides which upon reduction and hydrolysis were converted to normal peptides.



In the same way DL-phenylalanyl-D-arginine was prepared from the azlactone (22) and D-arginine and DL-tyrosyl-D-arginine from the azlactone of *p*-acetoxy- α -acetamidocinnamic acid and D-arginine (26).

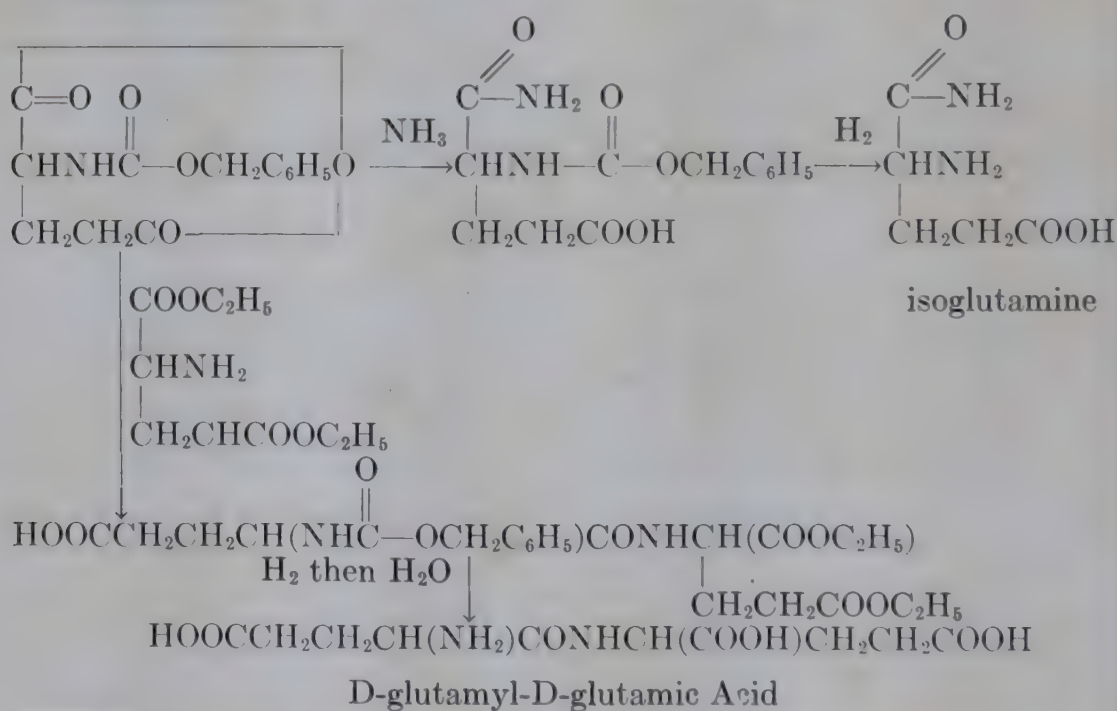
The low yield in the hydrogenation step was due to the fact that a separation of isomers was necessary since saturation of the double bond created a new asymmetric center.

5. Carbobenzoxy chloride synthesis*

The introduction of the carbobenzoxy radical as the masking group in peptide synthesis constituted one of the most important advances in this field. The reagent, carbobenzoxy chloride, was prepared according to the following equation (45).



Bergman and Zervas (24) showed that the anhydride of carbobenzoxy-D-glutamic acid reacted with amino compounds through the alpha carboxyl group, since isoglutamine resulted after reaction with ammonia.

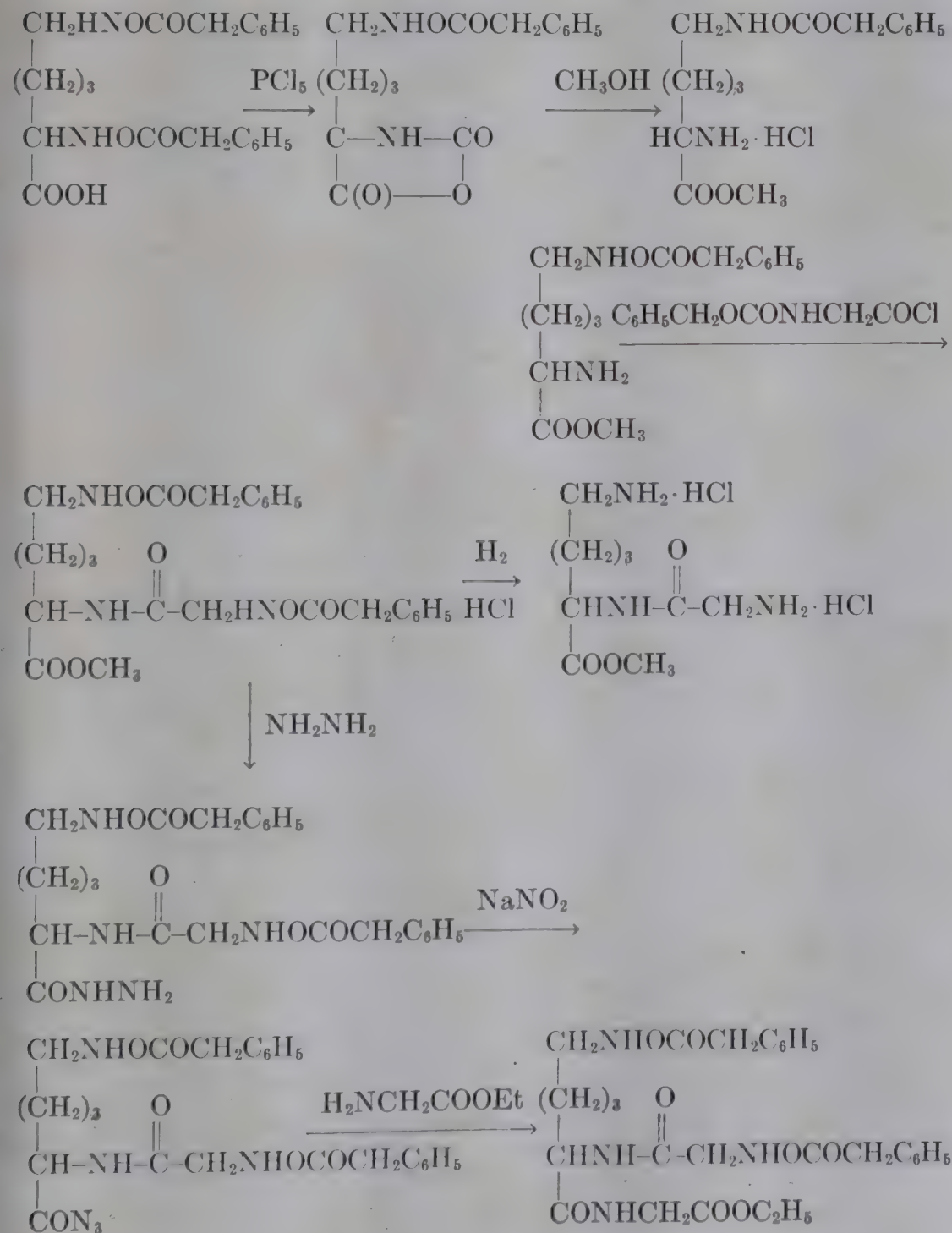


* Chantrenne (49a) has synthesized peptides by reacting the acyl phosphate of an amino acid with the amino group of a free amino acid. It is noteworthy that this reaction takes place under physiological conditions of pH, temperature and concentration, namely, at pH 7.4, 37° and 0.01 M concentrations. Peptides that have been prepared in this manner are glycylglycine, glycytryptophan, and glycylglycyl tryptophan.

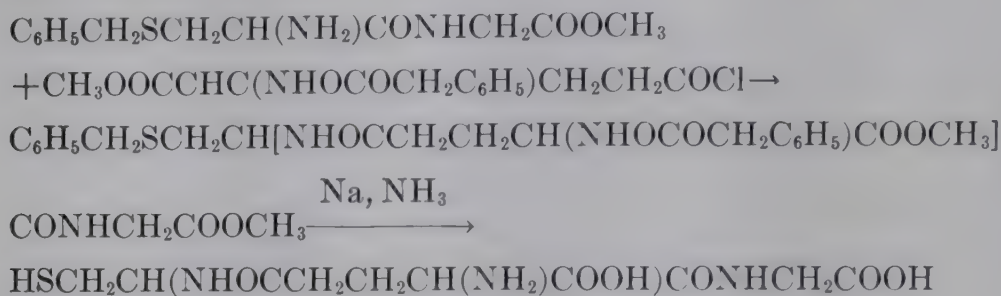
In carrying out the synthesis carbobenzoxyglycyl chloride is reacted with disilver phenylphosphate to form the phenyl carbobenzoxyglycyl phosphate.



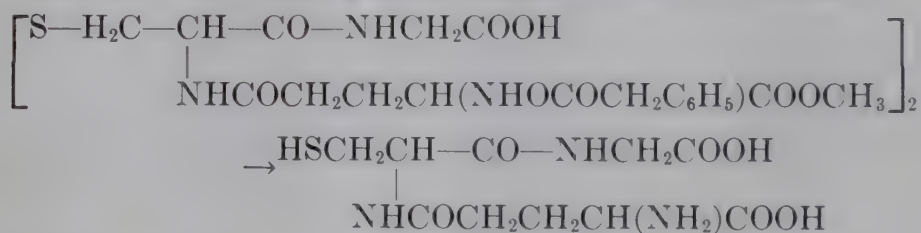
Peptides of lysine were also prepared (25). The dicarbobenzoxy derivative of lysine which was obtained in quantitative yield gave an anhydride on treatment with phosphorus pentachloride. This anhydride was converted to ϵ -carbobenzoxy lysine methyl ester hydrochloride in methanolic hydrogen chloride. The following reactions serve to illustrate how glycyl residues may be linked on either the carboxyl or amino groups of L-lysine. Catalytic reduction of α -carbobenzoxyglycyl- ϵ -carbobenzoxy-L-lysylglycine ethyl ester would give the corresponding tripeptide.



Glutathione was synthesized by du Vigneaud and Miller (262) by a carbobenzoxy synthesis. The carbobenzoxy group and the benzyl group were removed with sodium and liquid ammonia. The over-all yield was 27%.

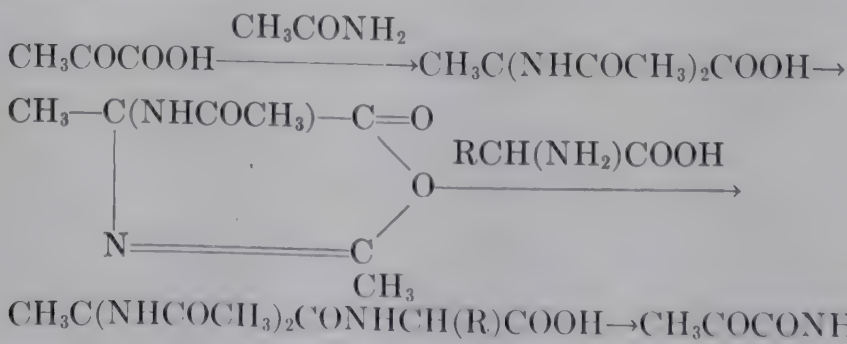


Similarly Harington and Mead (144) reduced carbobenzoxy- γ -glutamylcysteylglycine with phosphonium iodide to effect cleavage of the carbobenzoxy group and the disulfide linkage.



6. α -Keto acid synthesis

Shemin and Herbst (238) developed a method of peptide synthesis based on earlier work of Bergmann and Grafe (21). The latter authors prepared pyruvyl derivatives of amino acids according to the following scheme. Shemin and Herbst converted pyruvyl-alanine ($\text{R} = \text{CH}_3$) to the corresponding oxime and hydrogenated the latter to alanylalanine with the aid of a platinum oxide catalyst. The oxime of pyruvylphenylalanine reduced to DL-alanylcyclohexylalanine.



The method is of limited application, suffering from the disadvantage of giving a racemic peptide and apparently cannot be used with aromatic amino acids.

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Chapter IIIB

THE SYNTHESIS OF LABELED ALPHA AMINO ACIDS*

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I. INTRODUCTION

AMONG the newer investigational methods available to the biochemist is the use of rare or artificial isotopes as tracers. The basis of the method is the fact that two isotopes of an element are so nearly identical in their chemical properties that in biological work the differences are not very important. As an example, glycine ($H_2NCH_2CO_2H$) can be prepared in such a way that the carboxyl group contains carbon whose content of the rare isotope C^{13} is greater than normal. Then the metabolic fate of the carboxyl carbon atoms of the glycine when administered to a living organism can be followed by ascertaining in which of the compounds present in the organism an abnormally high concentration of C^{13} appears. The finding of extra C^{13} in a compound means that the organism synthesizes the compound by a method which utilizes, directly or indirectly, the carboxyl group of glycine. If glycine is prepared with the alpha carbon position labeled, the fate of this part of the molecule can be learned, and by using a rare isotope of nitrogen, the amino group can be followed.

A rare hydrogen isotope too, can be used to label the glycine. Although this may be done in certain cases in order to follow the hydrogen, the purpose is more commonly to use the hydrogen as a tracer for the carbon to which it is attached. Thus, glycine in which

* Completed April 1948.

the hydrogen attached to the alpha carbon atom is enriched in deuterium can be administered to an animal and then located in the tissues by means of deuterium analysis, provided, of course, that the deuterium has not become separated from the carbon. This requirement, that the hydrogen be stably bound to the atom for which it serves as a tracer, places certain limitations upon its use in labeling compounds. It is not possible to use hydrogen as a tracer for the amino group of glycine for instance, because in the body of an experimental animal the deuterium of the amino group will exchange with the hydrogen of water and the amino hydrogen will rapidly approach the isotopic composition of ordinary hydrogen. The amino group of the glycine will then no longer be distinguishable from any other amino group.

TABLE I
ISOTOPES USED TO LABEL AMINO ACIDS

Element	Mass	Type	Primary Purchasable Forms ^a
Carbon	11	Radioactive	Prepared in cyclotron immediately before use.
	13	Stable	Cyanide, methyl iodide, carbonate
	14	Radioactive	Carbonate, methanol, cyanide
Hydrogen			
Deuterium	2	Stable	Gaseous hydrogen, water.
Tritium	3	Radioactive	Prepared in cyclotron
Nitrogen	15	Stable	Ammonium-N ¹⁵ nitrate, potassium phthalimide
Sulfur	35	Radioactive	Sulfuric acid, sulfide
Iodine	131	Radioactive	Iodide

^a Rapidly being increased in number.

Tracer isotopes may be of either the stable or radioactive variety. The concentration of a stable tracer in a specimen under observation is determined by a method of measuring mass differences, such as density measurement or mass spectrometer assay; an unstable tracer is determined with a Geiger-Müller counter or other device for measuring radioactivity.

Satisfactory sources of tracer isotopes are available for all the elements of major biochemical importance with the exception of oxygen. It is difficult to enrich oxygen in stable isotopes to a useful degree, and no suitable radioactive isotope has been prepared. Table I summarizes the characteristics of the isotopes which have been used to label alpha amino acids.

The synthesis of labeled molecules must be conducted under certain restrictions which limit the choice of synthetic methods: 1. The isotopic starting materials are expensive, and the method

of choice will be the one which gives the best yield based on isotope. Since the yield decreases with each step in a sequence of reactions, a scheme is sought in which the isotope is introduced as late as possible. 2. The number of primary forms in which the concentrated isotopes can be purchased is limited. 3. If the nature of an experiment requires that a particular position in a molecule be labeled rather than any other position, the choice of synthetic method will be further narrowed. Thus, a method satisfactory for preparing glycine labeled with carbon in the carboxyl group is not necessarily suitable for preparing glycine labeled at the alpha carbon atom. 4. With certain radioactive isotopes, the amount of activity necessary to conduct a series of experiments within the activity limits required by tracer experiments may be contained in only a few hundred milligrams of product; thus, the synthetic methods should be adaptable to operation with small quantities of material (a gram or less). 5. The health hazard presented by radioactive substances limits the amount of activity which can be prudently handled in an ordinary laboratory.

II. INTRODUCTION OF DEUTERIUM AND TRITIUM (D₂ AND T₂) INTO AMINO ACIDS

The synthesis of amino acids labeled with isotopic hydrogen is accomplished by methods which involve either the direct exchange of normal for isotopic hydrogen or the reduction of an unsaturated linkage with isotopic hydrogen.

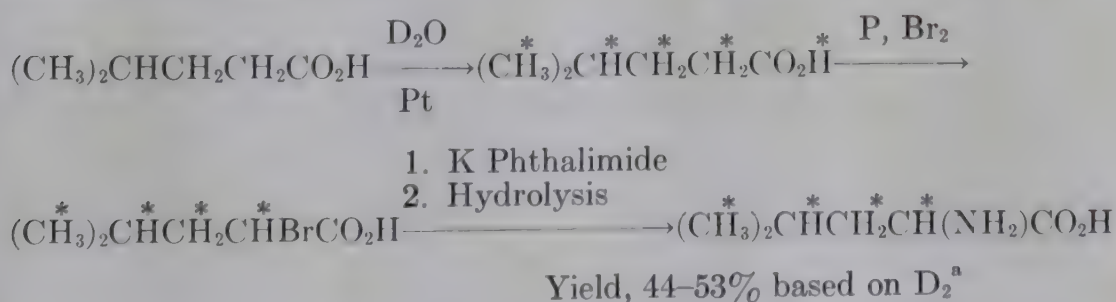
1. Exchange Reaction

The exchange reaction has the advantage that by its use many compounds which are difficult to synthesize can be easily labeled. The conditions necessary for exchange vary with the compound; in general, hydrogen attached to an aromatic carbon atom exchanges more readily than when bound to an aliphatic one, and the lability is increased by the presence of an activator, such as an ortho hydroxyl group. On the other hand, the rate of exchange of hydrogen bound to an aliphatic carbon atom can be increased by the presence of an acid strengthening group such as an α -unsaturation. By varying the experimental conditions a variety of compounds can be labeled with isotopic hydrogen. Optically active amino acids are racemized when the alpha hydrogen is replaced.

The following amino acids have been prepared by the exchange of hydrogen with strong (80–90%) deuterio- or tritiosulfuric acid: *deuterio-DL-phenylalanine* (1), *deuterio-DL-alanine* (2, 3), *deuterio-DL-leucine* (2, 3), and *tritio-DL-phenylalanine* (4).

Isotopic hydrogen has also been introduced into amino acids by the use of more dilute acid (20% hydrogen chloride or sulfuric acid in heavy water) by prolonged heating at elevated temperature. Isotopes introduced in this way can be removed under the same conditions by reverse exchange if the solvent is ordinary water. This consideration places a limitation on the use of amino acids labeled with dilute acid in experiments where they will be subjected to the action of hot acid (as in the hydrolysis of a protein). Concentrated sulfuric acid, on the other hand, is capable of causing exchange of hydrogen too stably bound to be removed by heating with 20% acid, and compounds labeled in this way can be used with less danger of loss of tracer caused by subsequent chemical manipulation. By the use of dilute acid in heavy water the following compounds have been prepared: *deuterioglycine* (3), *deuterio-L-proline* (3), *deuteriocystine* (3, 5), *deuteriotyrosine* (3), *deuterio-L-glutamic acid* (3), *deuterioarginine* (5), and *deuteriolysine* (5). After several days' heating the extent of exchange is small in all cases.

In the presence of platinum black many compounds will exchange hydrogen with heavy water. Amino acids themselves do not exchange well, but the method can be used to prepare labeled starting materials from which amino acids can be synthesized. Thus, *deuterioisocaproic acid* has been made by exchange and used to prepare *deuterio-DL-leucine* (6):



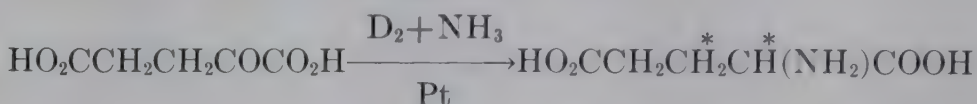
Leucine labeled with both deuterium and N¹⁵ has been prepared by the same reaction sequence, using potassium phthalimide-N¹⁵ (Section III). By the same sequence of reactions *deuterio-DL-valine* has been prepared from labeled isovaleric acid (6).

^a The asterisk is used to designate the location of the tracer isotope.

2. Reduction of an Unsaturated Linkage

The hydrogenation of an unsaturated linkage as a method of introducing isotopic hydrogen has the advantage that an isotope introduced in this way is ordinarily stably bound.

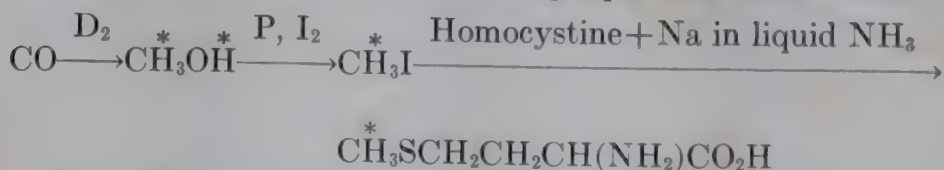
A particularly convenient reduction procedure is the Knoop reaction, in which an α -keto acid is converted to an amino acid by treatment with hydrogen and aqueous ammonia in the presence of a platinum or palladium catalyst. By substituting isotopic for ordinary hydrogen in the procedure, *DL*-glutamic acid- α,β -d can be prepared (7) from α -ketoglutaric acid:



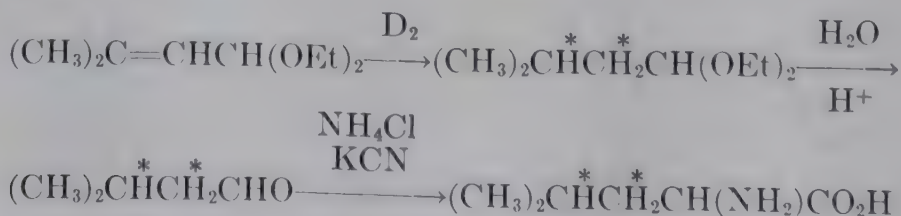
A certain amount of isotope can also be introduced into the glutamic acid if heavy water is used as the solvent and ordinary hydrogen is used for the reduction. This procedure gives a different distribution of isotope in the molecule. The Knoop reaction has also been used to prepare glutamic acid labeled with both deuterium and N^{15} (Section III).

Deuterio-DL-alanine has been prepared (8) by the Knoop reaction, with gaseous deuterium.

Deuteriomethanol has been prepared by catalytic reduction of carbon monoxide and converted to deuteriomethyl iodide. The methyl iodide was then used to prepare labeled *methionine* (9):

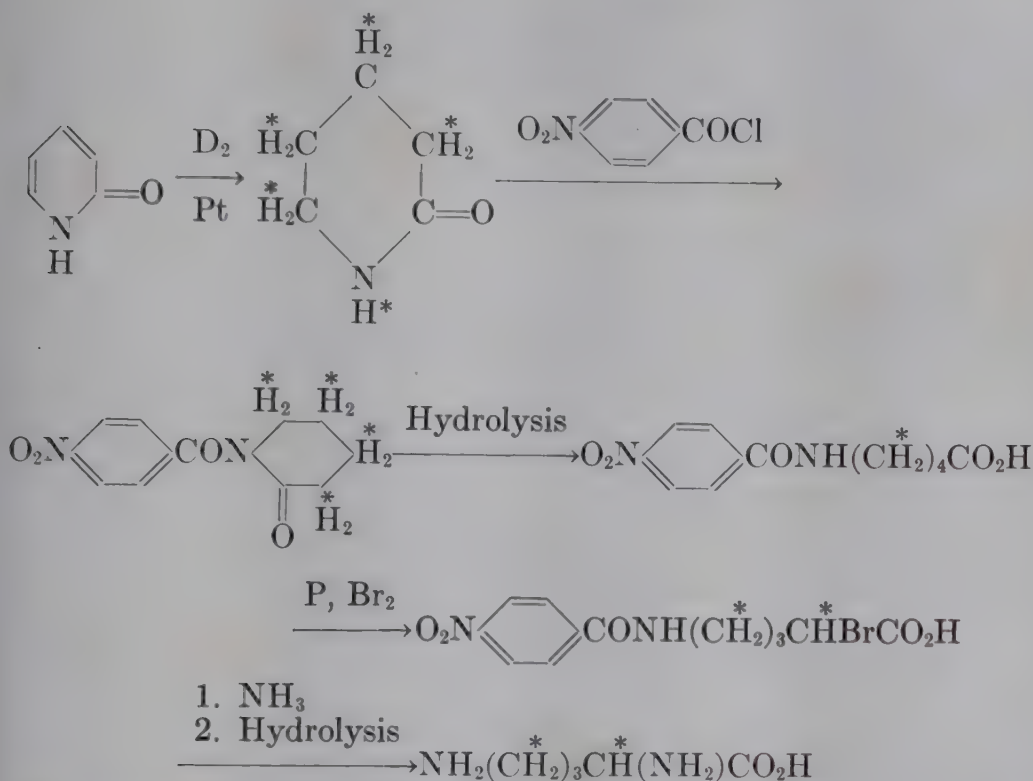


Reduction of an unsaturated acetal provides a route to a labeled aldehyde which can then be converted to an amino acid. In this way *DL-leucine*- β,γ -d has been prepared (10) from isopentenyl diethyl acetal:



In the same way, *DL-valine*- β,γ -d has been prepared (10) from isobutenal diethyl acetal.

Catalytic reduction has been employed to convert α -pyridone to α -piperidone which was then used to prepare *DL*-ornithine- $\alpha,\beta,\gamma,\delta$ -d (11).



Labeled α -pyridone has also been used to prepare proline labeled with both deuterium and N^{15} (see Section III; amino acids labeled with N^{15}).

Deuteriocyclohexanone prepared by the catalytic reduction of phenol can be used as a starting point for the preparation of doubly labeled lysine (Section III). Attempts to prepare deuteriocyclohexanone by direct exchange between cyclohexanone and deuterium oxide in the presence of platinum fail to give a satisfactory yield.

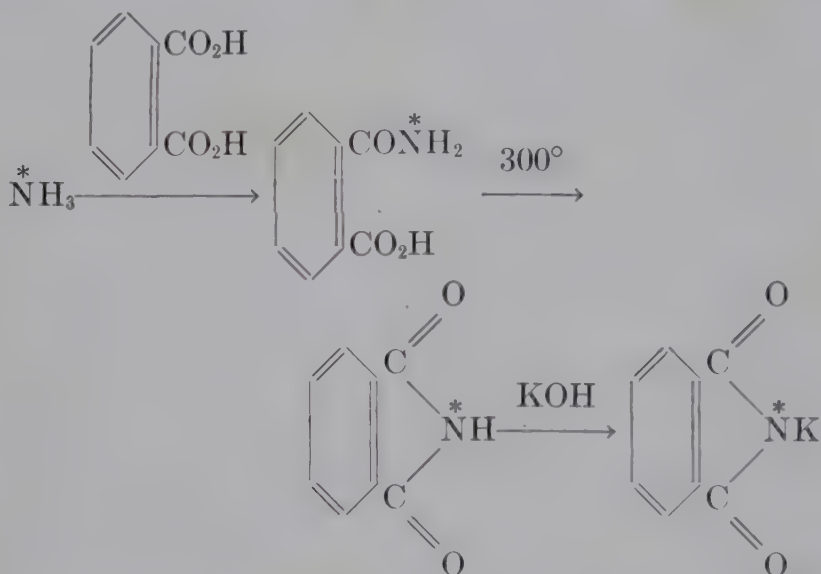
III. INTRODUCTION OF N^{15} INTO AMINO ACIDS

In general, amino acids do not exchange their α -amino nitrogen with other nitrogenous compounds under ordinary conditions (14), and exchange reactions cannot be used to introduce the tracer. Neither is the classical reaction between an α -halo acid and ammonia a convenient route, because a large excess of ammonia is required, a feature which makes for inefficient utilization of the enriched starting material. Several synthetic methods have been successfully adapted to tracer work, however; of these the two most

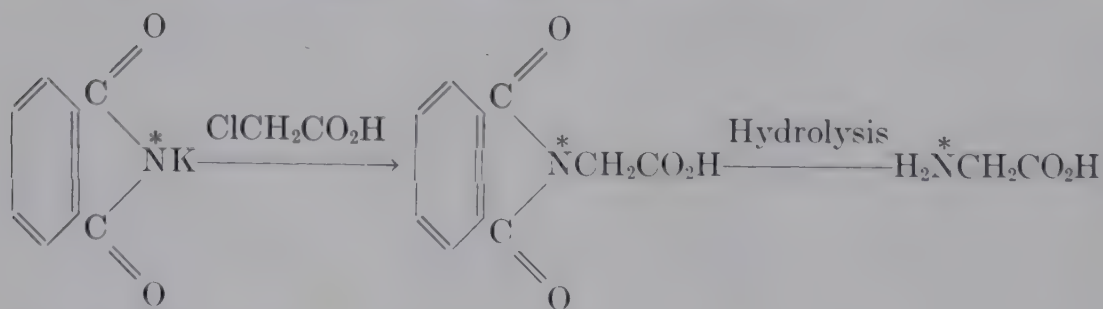
generally applicable are the Gabriel and the Knoop synthesis. The Strecker reaction also finds application.

1. Gabriel Synthesis

Isotopic ammonia can be converted to potassium phthalimide, practically without loss (13):

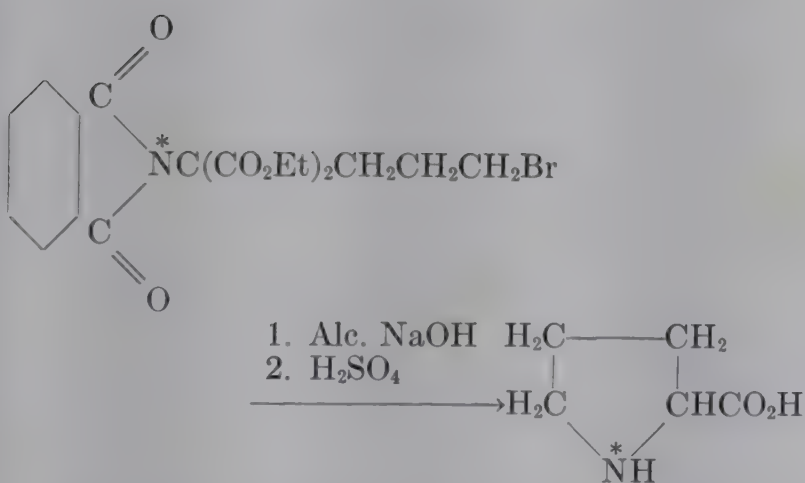
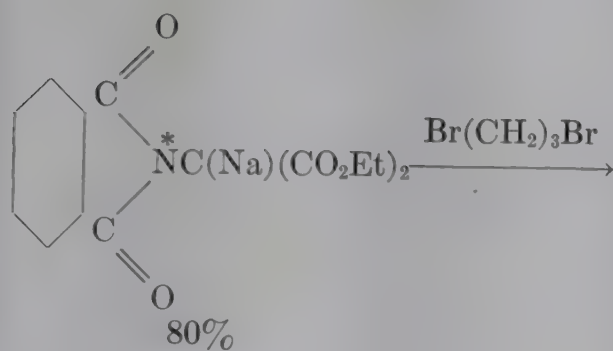
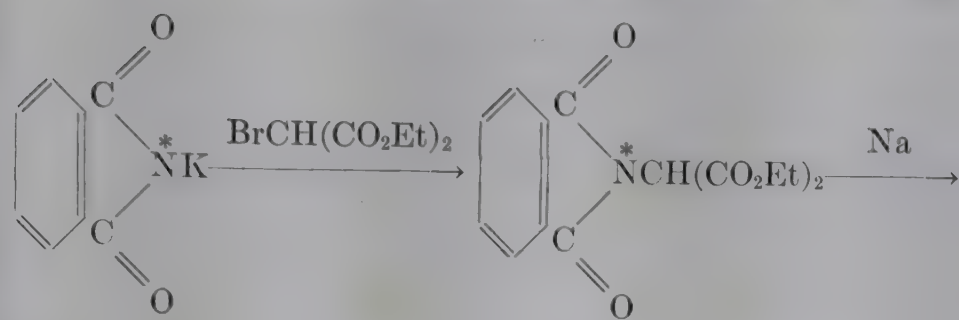


The labeled phthalimide can then be converted to a variety of amino acids by condensation with the proper α -halo acids. Thus *glycine*- N^{15} (13, 15, 16) has been prepared from chloroacetic acid in 89% yield, based on potassium phthalimide:



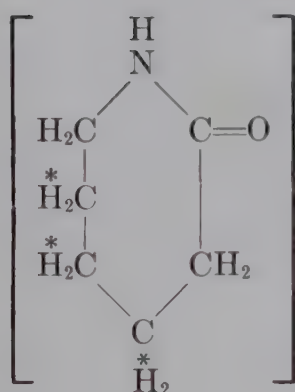
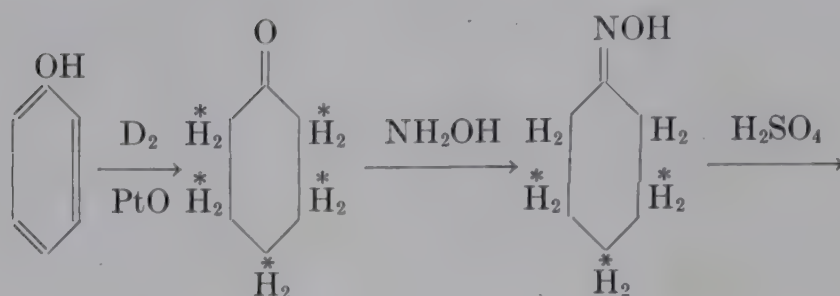
By this use of the Gabriel synthesis *DL*-lysine- N^{15} (13) in 72% yield, based on phthalimide and *DL*-serine- N^{15} (17, 15) have also been prepared. *D*-serine- N^{15} and *L*-serine- N^{15} were prepared (15) from the synthetic racemic mixture.

The modified Gabriel synthesis employing diethyl sodium phthalimidomalonate has been applied (15) to the preparation of *DL*-proline- N^{15} :

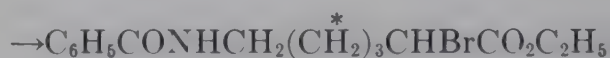
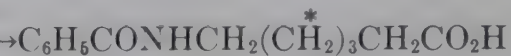


The Gabriel synthesis can be used to prepare an amino acid labeled with both nitrogen and hydrogen by employing an α -halo acid labeled with isotopic hydrogen. By this modification, *deuterio-DL-leucine-N¹⁵* has been prepared (13) from deuterioisocaproic acid with the use of the same sequence of reactions described in Section II for the singly labeled compound. The D and L forms of *deuterio-leucine-N¹⁵* have been isolated (18, 19) from the synthetic racemic mixture.

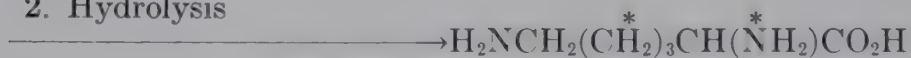
Deuteriocyclohexanone, prepared by the catalytic reduction of phenol, has been used (20) to prepare *DL-lysine- β,γ,δ -d-N¹⁵*



1. Hydrolysis
2. Benzoylation



1. Phthalimide - $N^{15} + CuO$
2. Hydrolysis



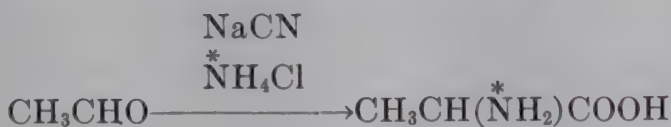
Labeled *L*-lysine was isolated from the racemic mixture.

2. Knoop Reaction

The use of the Knoop reaction for the preparation of amino acids labeled with isotopic hydrogen has been discussed in Section II. The reaction can be used equally well to introduce isotopic nitrogen by substituting ammonia- N^{15} for ordinary ammonia in the procedure. In this way the following amino acids have been prepared from the corresponding keto acids: *DL-alanine- N^{15}* (13), *DL-phenylalanine- N^{15}* (13), *DL-tyrosine- N^{15}* (13), *DL-norleucine- N^{15}* (13), *DL-aspartic acid- N^{15}* (13, 15), *DL-glutamic acid- N^{15}* (13), and *DL- γ -phenyl- α -amino- N^{15} -butyric acid* (2). The yields range from 40 to 85%, based on total ammonia taken; most of the ammonia remaining unconverted is recoverable as such from the liquors. From racemic mixtures, *L-glutamic acid- N^{15}* (15) and the D and L forms of *γ -phenyl- α -amino- N^{15} -butyric acid* (21) have been prepared.

An amino acid labeled with both hydrogen and nitrogen can be prepared by the Knoop reaction if isotopic hydrogen and $N^{15}H_3$ are used together in the procedure. By this device, α -ketoglutaric acid has been converted (22) to *DL-glutamic acid- α,β -d- N^{15}* .

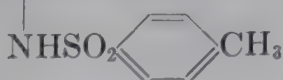
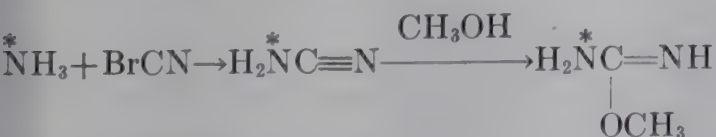
By a modification of the *Strecker* procedure, acetaldehyde has been converted to *DL-alanine-N*¹⁵ by treatment with sodium cyanide in the presence of isotopic ammonium chloride (15).



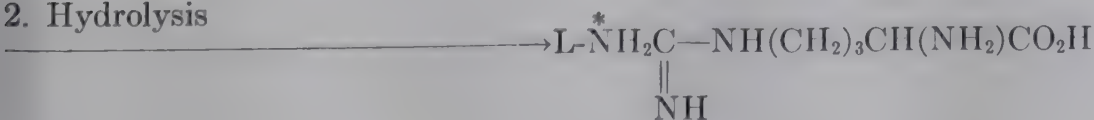
L-alanine- N^{15} was isolated from the synthetic racemate.

In addition to these general types, a few specialized syntheses have been carried through with isotopic materials.

L-arginine- N^{15} labeled in the amidine group has been prepared (16) by the following sequence:

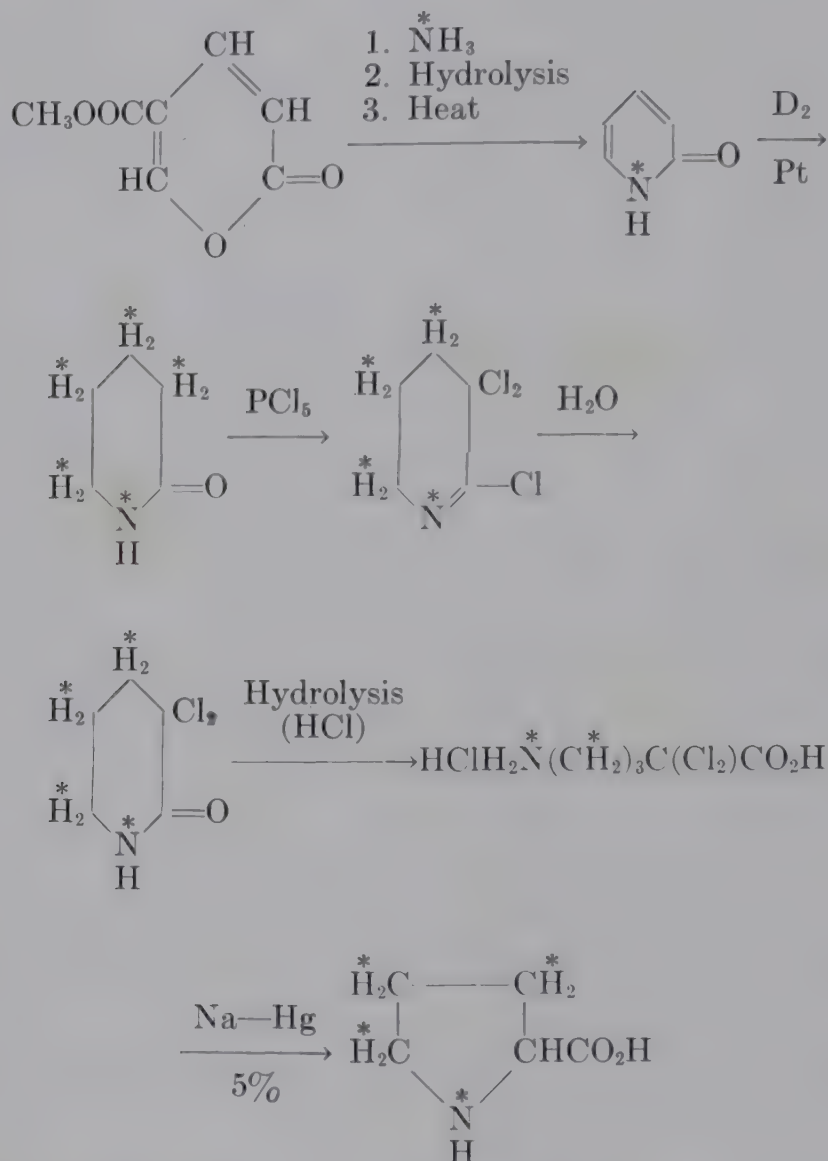


2. Hydrolysis



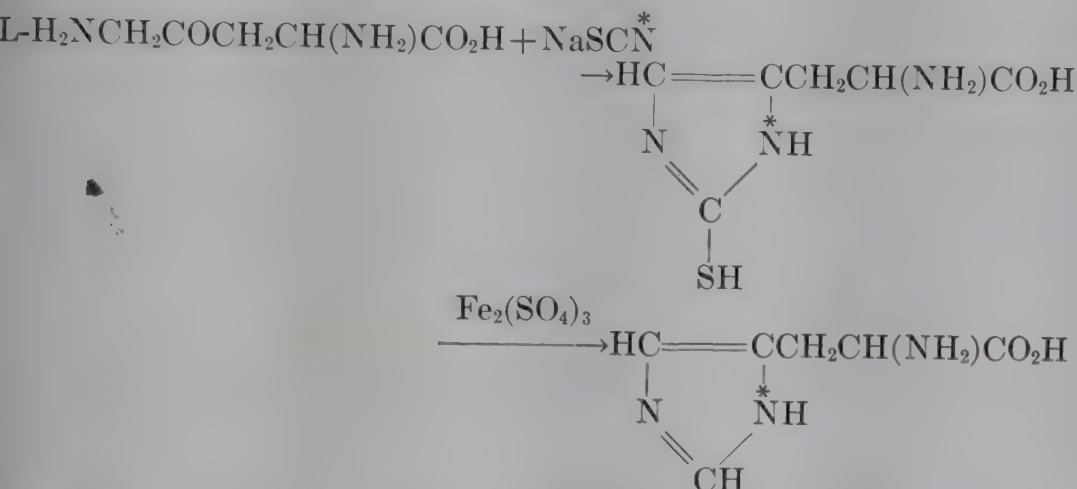
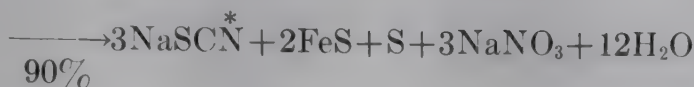
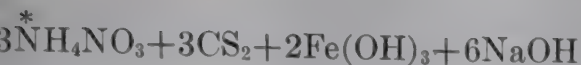
Two nitrogen atoms in the guanidine group are rendered indistinguishable by tautomerism.

DL-proline-3,4,5-*d*- N^{15} has been prepared (12) by the following sequence:



L-proline-3,4,5-*d*- N^{15} was isolated from the racemic mixture.

L-histidine-1- N^{15} has been prepared (23) by reaction of sodium thiocyanate- N^{15} with γ -ketoornithine, followed by treatment with ferric sulfate of the thiolhistidine so formed:



The yield was 17% based on thiocyanate.

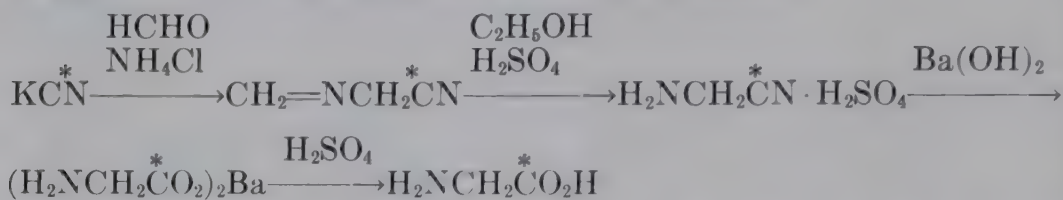
IV. INTRODUCTION OF C¹³ AND C¹⁴ INTO AMINO ACIDS

The preparation of amino acids labeled with isotopes of carbon (C¹³ or C¹⁴) requires the use of a greater variety of synthetic methods than does the synthesis of these compounds labeled with other isotopes; the usual isotopic starting material is either cyanide or carbonate.

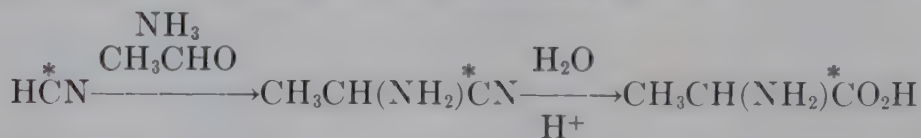
The isotope C¹¹, with a half-life of 21 minutes, has not been used to label an amino acid, although it has been used for tracer studies in other types of compounds of biological interest. Because of the nature of the operations involved, it would be difficult to synthesize a labeled amino acid, carry out an experiment and make radioactivity measurements quickly enough to avoid losing most of the original activity by decay.

Cyanide, in which form isotopic carbon can be purchased or can be prepared from isotopic barium carbonate (24, 25), is a particularly convenient material with which to begin the synthesis of an amino acid. It can be used directly in the Strecker reaction, or it can be treated with an aliphatic halogen compound to form a nitrile. The nitrile can then be hydrolyzed, and the resulting carboxylic acid converted to an amino acid by one of the standard methods.

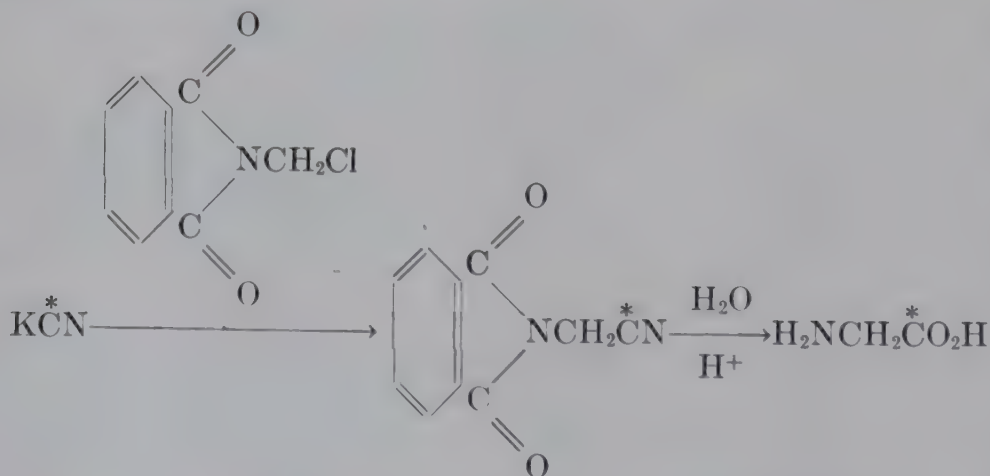
Thus, the methyleneaminoacetonitrile synthesis, a derivative of the Strecker procedure, has been used (25) to convert formaldehyde to glycine-1- C^{14} in 50% yield, based on cyanide.



Acetaldehyde has been converted (25, 26) by the Strecker synthesis to *DL*-alanine-1- C^{14} . The yield, based on barium carbonate from which the cyanide was prepared, is 35%.



Glycine-1- C^{13} has been prepared (27) in 81% yield, based on cyanide, by the reaction between chloromethylphthalimide and labeled cyanide, followed by hydrolysis of the phthalimidoacetonitrile so formed.



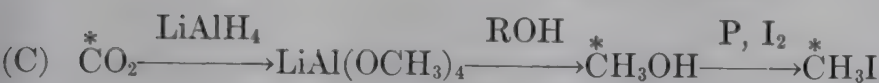
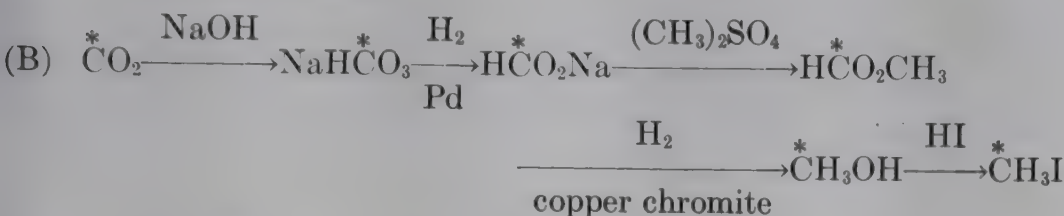
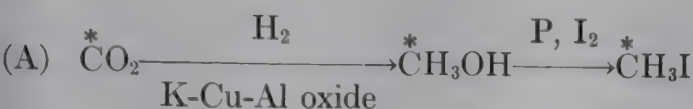
The same procedure has been used (26) to prepare *glycine*-1- C^{13} - N^{15} from chloromethyl phthalimide labeled with N^{15} .

A synthesis of *DL*-methionine- S^{34} - β,γ - C^{13} in which cyanide is used as the primary source of C^{13} is described in Section V.

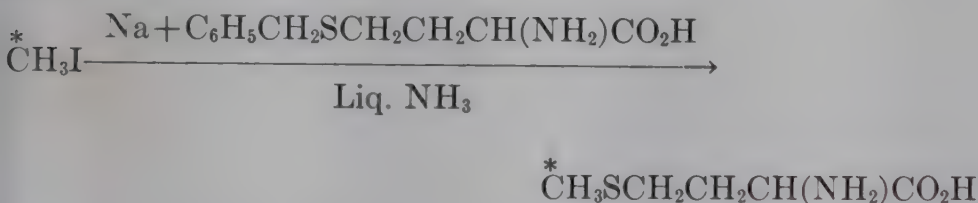
Practically all of the procedures for introducing isotopic carbon which do not employ labeled cyanide as the starting material begin with isotopic carbon dioxide (barium carbonate).

Carbon dioxide has been converted to methyl iodide by three semimicro techniques. The first (28) requires the use of high pressure equipment, but gives a yield of 84% against 76% for the second (29). The yield of methanol based on carbon dioxide is 81% by

the third method (30), which requires the least specialized equipment.



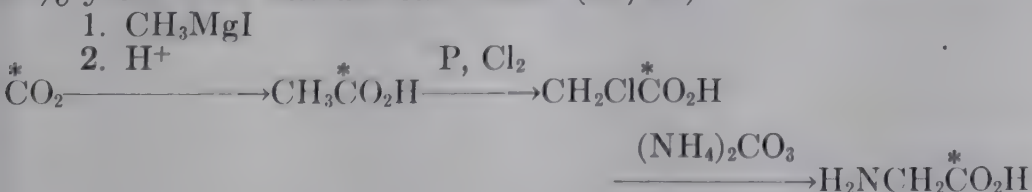
Methyl iodide- C^{14} so prepared can be used (29) to convert S-benzylhomocysteine to *L*-methionine-methyl- C^{14} in 84% yield, based on methyl iodide:



Methyl iodide- C^{14} can also be used (31, 32) to prepare *glycine-2- C^{14}* . The iodide is converted to the Grignard reagent, which is carbonated with ordinary carbon dioxide to form acetic acid-2- C^{14} . This is converted to chloroacetic acid and aminated. The yield is about 55% based on isotopic barium carbonate used as starting material.

The synthesis of *glycine-2- C^{14}* just described illustrates the use of the carbonation of a Grignard reagent to form a carboxylic acid. By the substitution of isotopic for ordinary carbon dioxide, this reaction can be adapted to the synthesis of carboxyl-labeled acids and is widely used for the purpose. Various procedures for converting carboxylic acids to amino acids can then be applied.

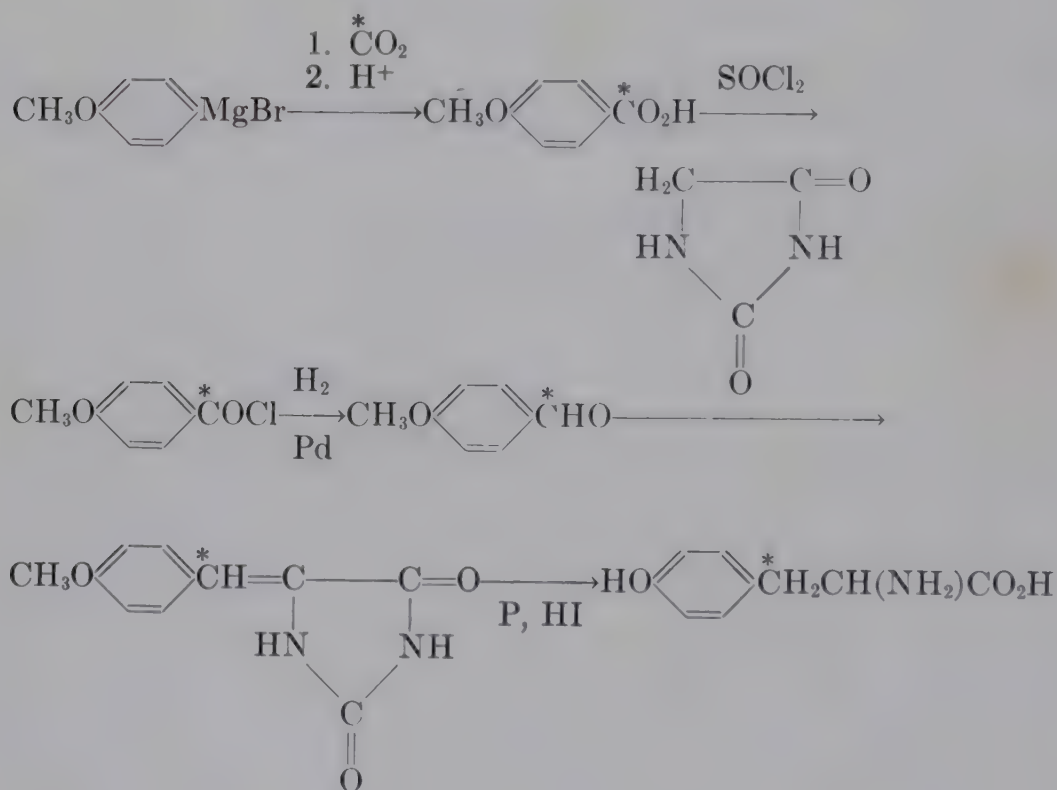
Thus carbon dioxide- C^{14} has been converted to *glycine-1- C^{14}* in 60% yield from barium carbonate (32, 33).



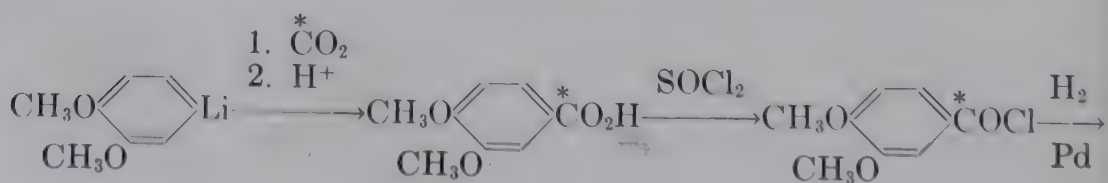
A somewhat better yield (69% based on barium carbonate) has been obtained (27) in the synthesis of *glycine-1- C^{13}* by conversion

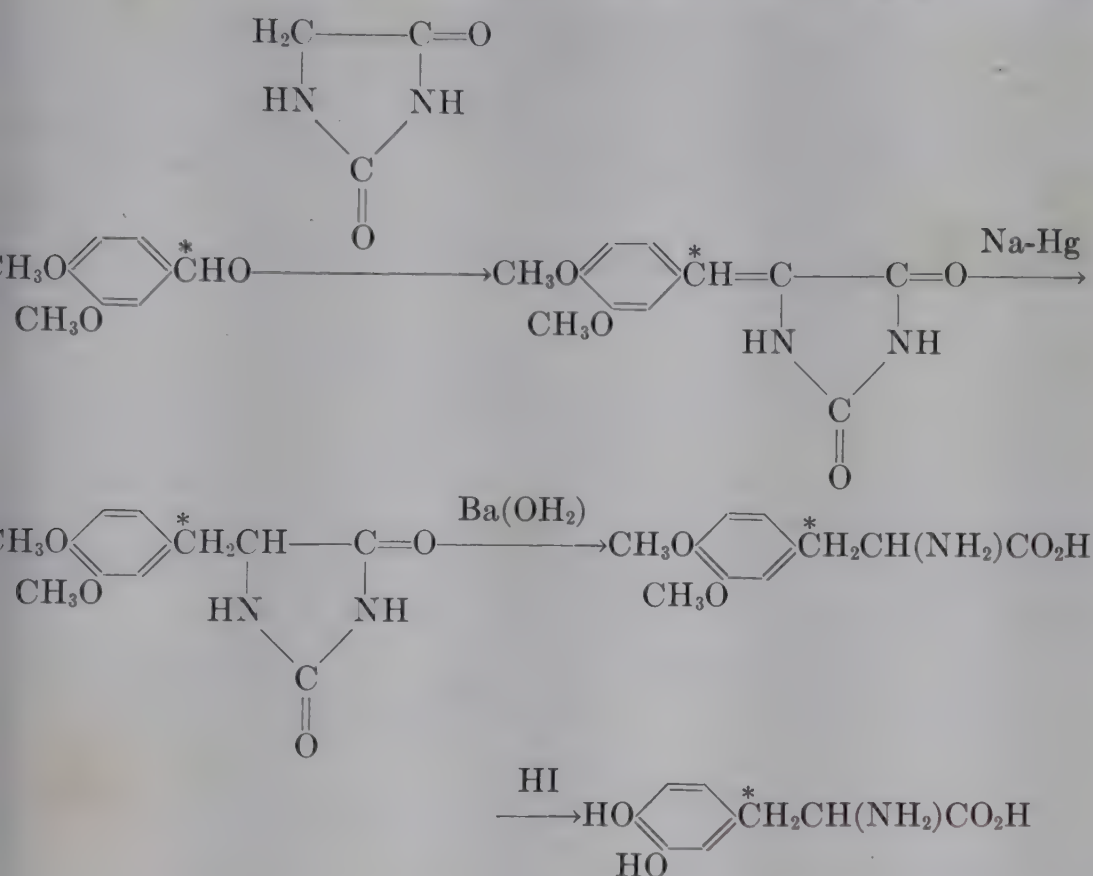
of the labeled acetic acid to ethyl bromoacetate and application of the Gabriel synthesis.

A carboxyl-labeled intermediate can sometimes be used to prepare an amino acid labeled in a position other than the carboxyl group. Thus *DL*-tyrosine- β - C^{14} can be prepared (34) in 19% yield (based on barium carbonate) from carboxyl-labeled *p*-anisic acid by conversion to *p*-anisaldehyde followed by condensation with hydantoin and reduction and hydrolysis of the *p*-methoxybenzalhydan-
dantoin so formed:

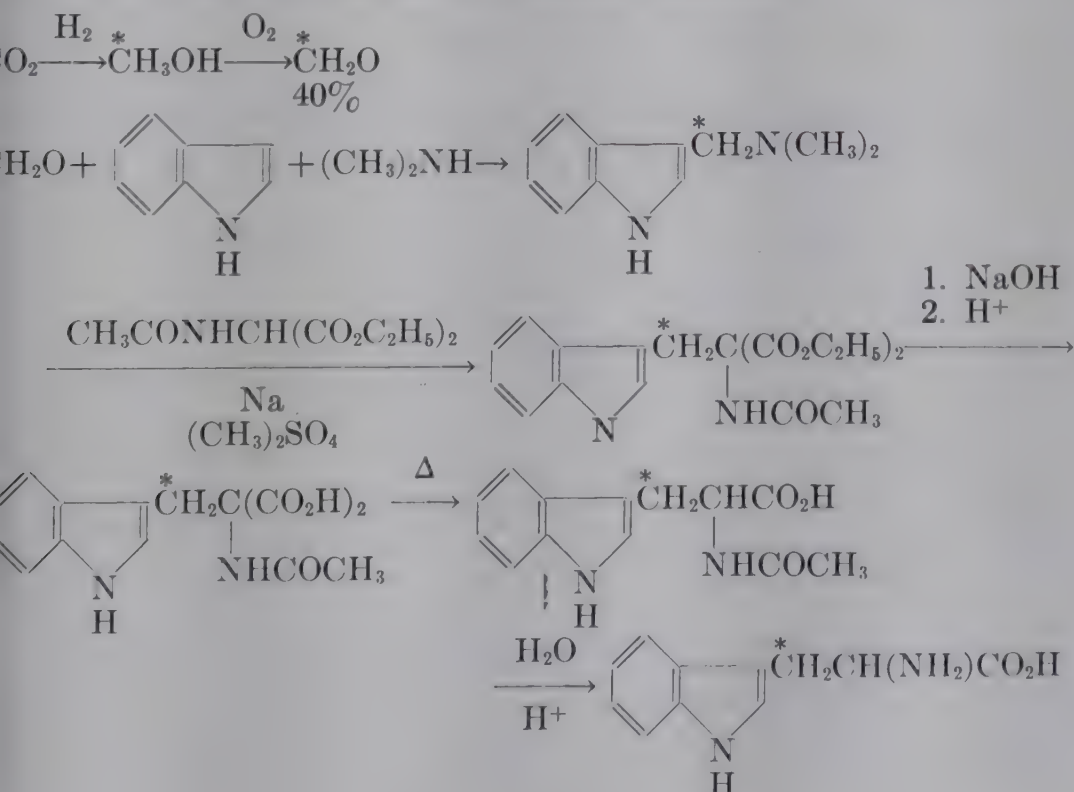


DL-3,4-Dihydroxyphenylalanine- β - C^{14} has been prepared (35) in 18% yield from barium carbonate by a scheme similar to that used for tyrosine, except that a lithium alkyl was used instead of a Grignard reagent in the initial carbonation (35):

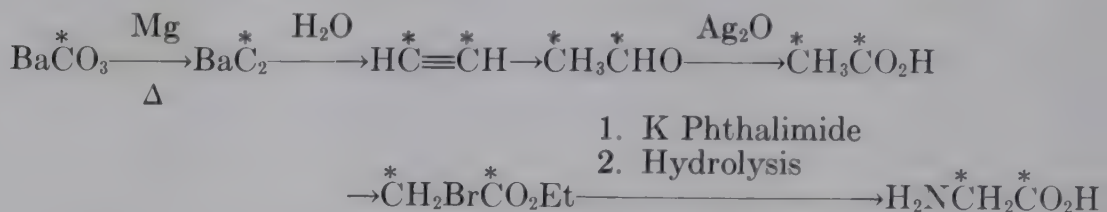




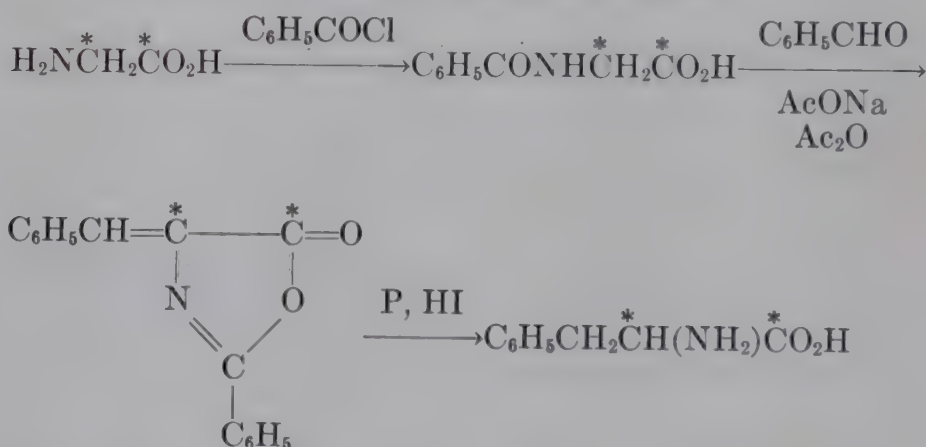
Formaldehyde, prepared by the oxidation of methanol, can be used (36) as the starting point for the synthesis of *DL*-tryptophan- C^{14} . The yield is 22% based on carbon dioxide.



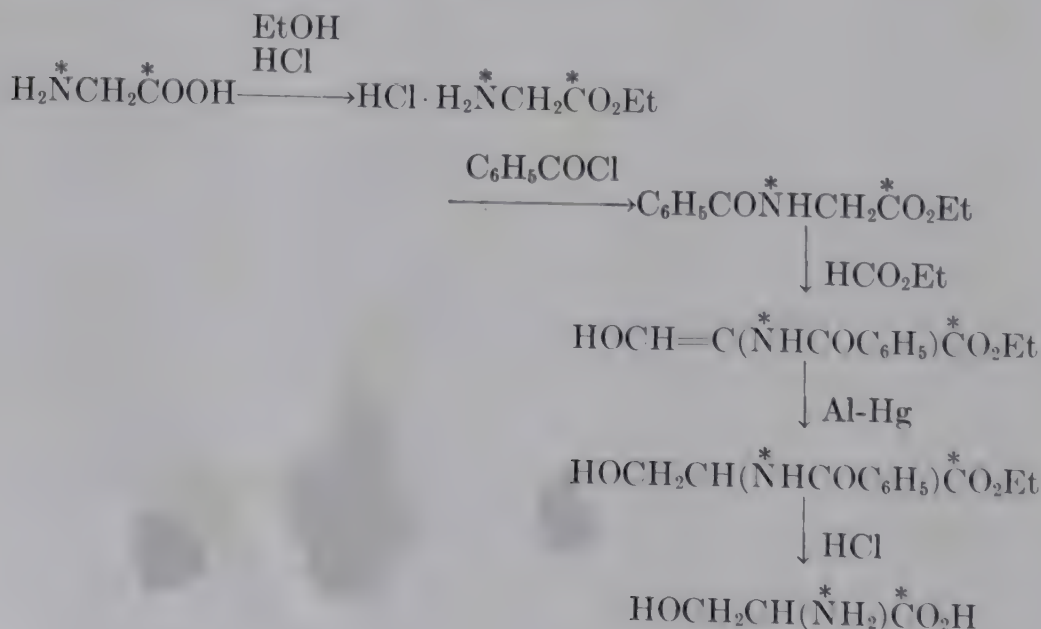
Acetylene prepared from isotopic barium carbide is a useful intermediate for the preparation of compounds labeled in two positions. *Glycine-1,2- C^{14}* has been synthesized from this intermediate by the following sequence (4):



Glycine itself has been used as an intermediate in the synthesis of other amino acids. *DL-phenylalanine- α ,carboxyl- C^{14}* has been prepared (4) from doubly labeled glycine- C^{14} by the azlactone synthesis. The overall yield based on glycine is 50%:



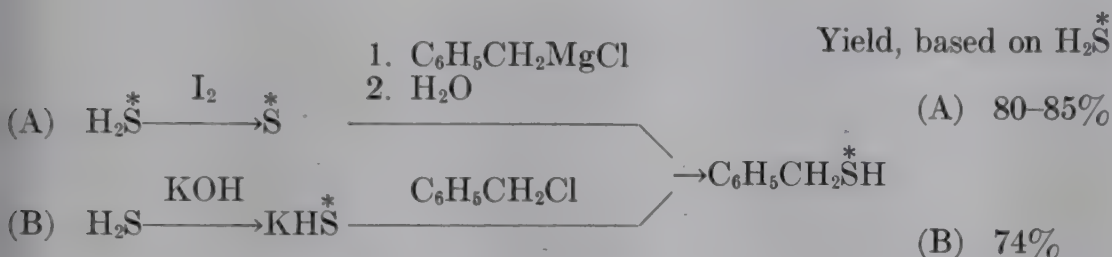
Labeled glycine has also been used as an intermediate in the synthesis (15) of *DL-serine-1- C^{13} - N^{15}* :



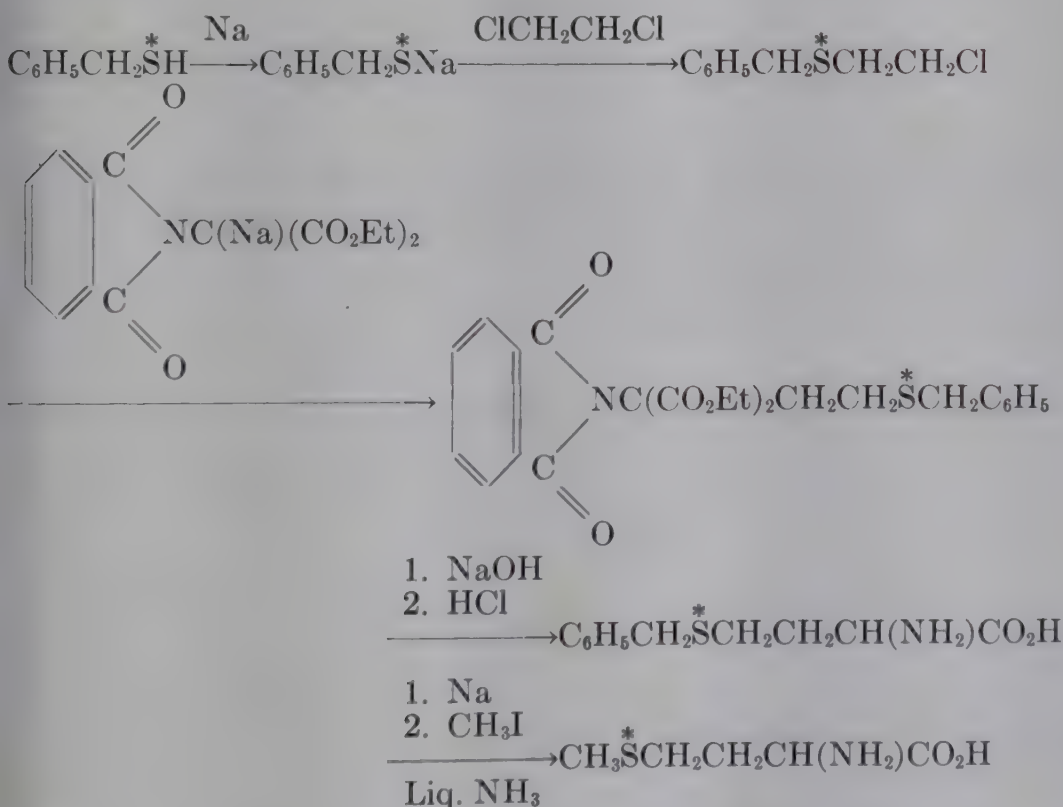
V. INTRODUCTION OF S³⁴ AND S³⁵ INTO AMINO ACIDS

In all of the methods so far applied to prepare amino acids with marked sulfur atoms, labeled benzyl mercaptan has served as an intermediate.

Labeled benzyl mercaptan can be prepared in two ways (37-40):



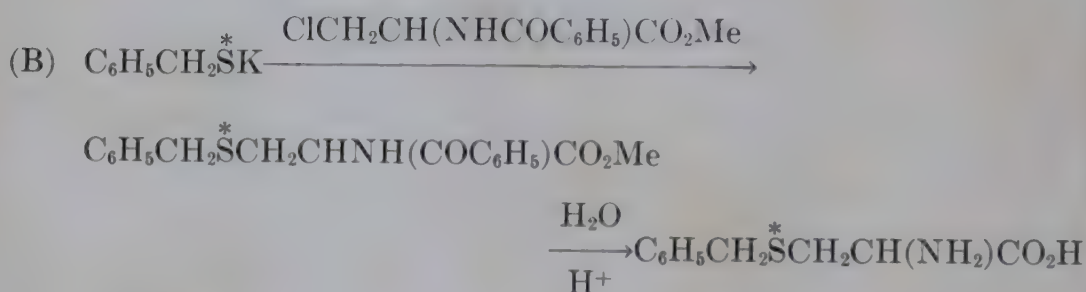
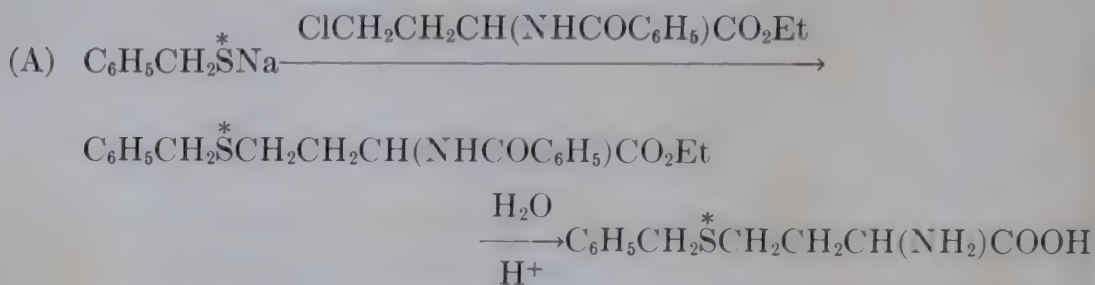
DL-methionine-S³⁵ has been prepared (38, 40) by continuing as follows:



The overall yield is about 20%, based on isotopic sulfur used to prepare the hydrogen sulfide.

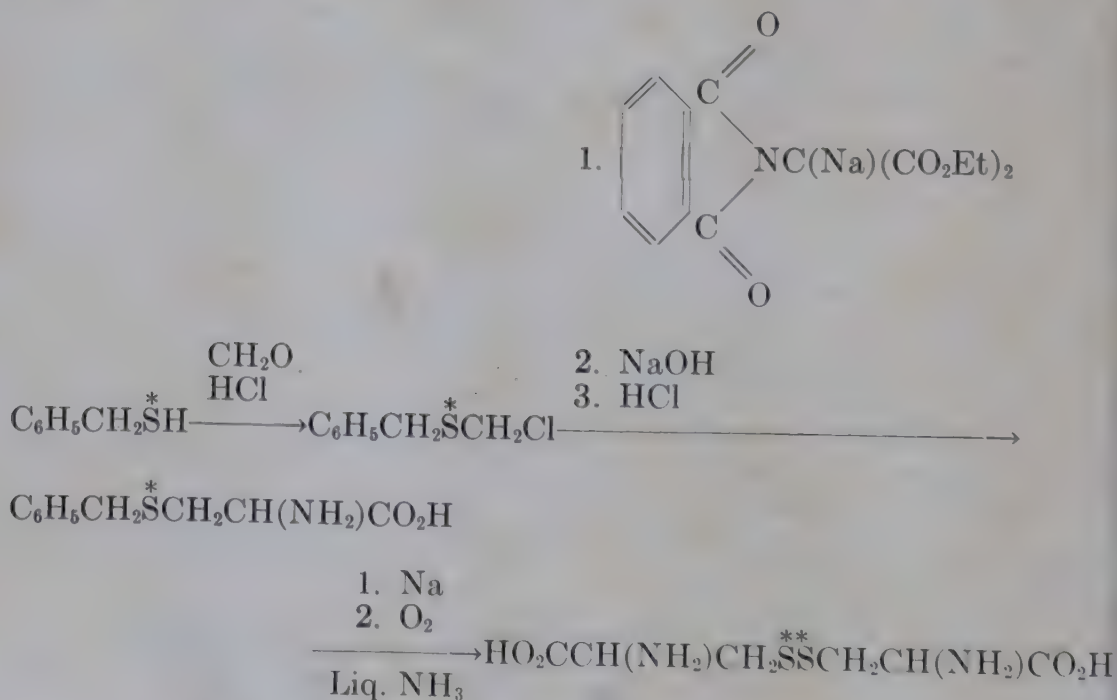
DL-homocystine-S³⁵ has been prepared (40) by the scheme just described for methionine, except that methyl iodide is not added in the last step; instead, oxygen is bubbled through the reduced solution. The yield is about 25% based on sulfur.

In an improved synthesis of methionine and cystine the benzyl mercaptan is condensed with ethyl γ -chloro- α -benzamido-*butyrate* (39) or methyl β -chloro- α -benzamido-*propionate* (41) respectively; hydrolysis of the condensation product gives benzylhomocysteine (A) or benzylcysteine (B):



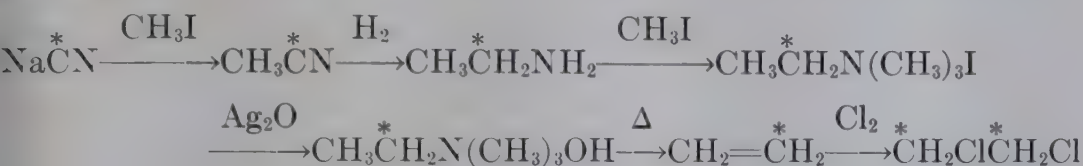
The benzyl group is then removed as before with sodium in liquid ammonia, followed by methylation or oxidation, respectively.

DL-cystine-*S*³⁵ has also been prepared (40) by the following method:



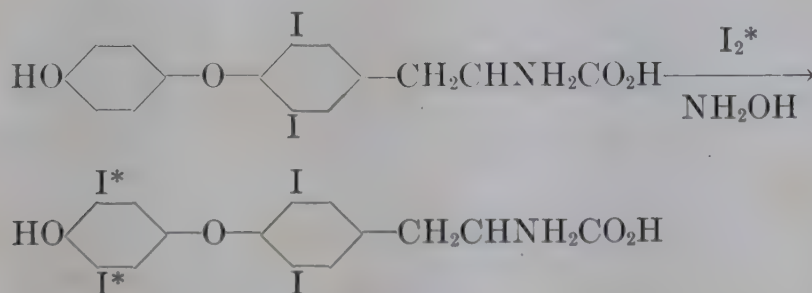
The yield is 17% based on benzyl mercaptan.

The doubly labeled *DL*-methionine- S^{34} - β , γ - C^{13} has been prepared (37) by the same method, employing ethylene chloride- C^{13} for the condensation with labeled benzyl mercaptan. The labeled ethylene chloride was prepared in 56% yield, based on cyanide, in the following way.



VI. INTRODUCTION OF IODINE¹³¹

In addition to preparation by extraction from the thyroid glands of animals given radioactive inorganic iodine, *thyroxine* has been prepared synthetically with two labeled iodine atoms (42) as follows:



The radioactive iodine was obtained as sodium iodide. By treatment of this compound with iodate, free iodine was prepared and this was used in the synthesis.

Labeled thyroxine has also been prepared (43) by direct exchange between thyroxine and radioactive iodide at pH 5.

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36. HEIDELBERGER, C. H., and TOLBERT, B. M.: unpublished work.
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Chapter IV

ISOLATION OF AMINO ACIDS*

MAX S. DUNN, PH.D. AND LOUIS B. ROCKLAND, PH.D.

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* Reviews have been given previously by Block (46), Calvery (54), Carter (56), and Vickery and Schmidt (273).

I. INTRODUCTION

THE PROTEINS of plants and animals are the principal sources of the naturally-occurring amino acids. Since proteins are essentially combinations of amino acid residues bound in peptide linkage, amino acids are readily formed by hydrolysis of proteins. It is possible to isolate nearly all of the 19 common amino acids from any protein, but from a practical standpoint, relatively few proteins are good sources of amino acids. Some of the critical factors to be considered are the availability and cost of the protein and the proportions of the constituent amino acids. Included among the protein materials which are considered to be the most acceptable sources of amino acids are those listed in Tables I and II. Current quotations and some specifications of protein materials obtainable from commercial concerns are given in Table I. The amino acid composition of these products is shown in Table II.

Amino acids occur in proteins, largely, as the so-called natural or L-isomers. Adequate procedures for the isolation of amino acids were desired originally because of their potential value in determining the amino acid composition of purified proteins. This objective appears to be of lesser importance at the present time because of the development, recently, of methods (particularly isotope dilution, chromatographic and microbiological procedures) with which in many cases, amino acids may be determined more conveniently and accurately than by gravimetric procedures.

It is desirable for other reasons that efficient, convenient and inexpensive methods be made available for the isolation of amino acids. The natural amino acids are probably superior to the synthetic (DL) forms in some, if not all, cases as substrates for growth of bacteria and other microbial forms as well as for clinical purposes. The demand for amino acids, singly and collectively, as pharmaceutical products has increased markedly in recent years because of their potential or demonstrated value in the alleviation of some types of liver diseases* (methionine), mental disorders† (glutamic acid), and other clinical conditions. In addition, the ingestion and injection of mixtures of pure amino acids or of protein hydrolyzates have been found to be efficacious in the treatment of inanition hypoproteinemia, burns, allergies, and other disorders (15, 101).

Glutamic acid is manufactured in large quantities as its mono-

* For reviews see Sahyun (221a, 221b).

† For reviews see Woods (283a) and Nutr. Rev. (200a).

sodium salt for use as a condiment.* The principal source of glutamic acid is wheat gluten although considerable quantities are isolated from Steffen's waste obtained in the manufacture of sucrose from the sugar beets. More than 100 patents have been issued on the isolation of glutamic acid, the first (U.S. 1,015,891) to Okeda and Suzuki of Tokyo on January 30, 1912 and the last (U.S. 2,373,342) to C. L. Royal of Toledo, Ohio on April 10, 1945. (Latest patent (U.S. 2,463,877) known to authors at time galley proof read was issued to F. A. Hoglen, March 8, 1949.) Some commercial sources of amino acids are listed in Table III.

II. ISOLATION PRINCIPLES

It has been common practice to isolate amino acids from particular proteins selected because of their availability, low cost and relatively high percentages of desired amino acid forms. Examples of such proteins and the amino acids isolated from them are human hair—cystine, wheat gluten—glutamic acid, corn gluten—leucine and tyrosine, casein—tryptophan, gelatin—arginine and proline, blood—arginine, histidine and lysine, and silk—alanine, glycine and tyrosine.

Certain amino acids are easily isolated by adjusting the pH of proteinhydrolysates to the isoelectric point (pH of minimum solubility). Cystine, tyrosine and leucine have been found to precipitate readily under these conditions although the initial products usually contain considerable quantities of other amino acids. The factors on which the isoelectric precipitation of amino acids depends include temperature, concentration, rate of crystallization, and solubility in the presence of hydrolytic degradation products, inorganic ions and other amino acids. In most instances, amino acids obtained in this manner are grossly impure and are not readily purified by simple recrystallization processes. It has been found advantageous to separate some amino acids as metal salts, heavy metal double salts and salts of inorganic or aromatic organic acids selected because of their relatively low solubilities or favorable crystallization tendencies. Examples of amino acid salts commonly utilized for this purpose are shown below:

Alanine	pyridoxate (dioxalatodipyridinochromiate) and azobenzene- <i>p</i> -sulfonate
Arginine	flavianate (1-naphthol-2,4-dinitro-7-sulfonate) and picrate
Aspartic acid	copper salt trihydrate

* For discussion see Crocker (69a), Caircross (53b), and Galvin (129a).

TABLE I
SOME COMMERCIAL SOURCES OF PROTEIN MATERIALS*

Protein Material						Com- mercial Concern Number
Type	Specifications (Approximate Average)				Cost (Approximate Average)	
	Total N	Mois- ture	Ash	Fat		
	per cent	per cent	per cent	per cent	per lb.	
Animal organs, defatted†					0.80-6.50	18
Casein, enzyme hydrolyzed	12.0	4	5.5		2.13	12
Casein, lactic	13.4	10	2.2	1.0	0.50	13
Casein, rennet	12.8	10	8	0.5	0.50	13
Casein, acid	13.8	11	2.5	0.5	0.27	14
Casein, high nitrogen	14.2	8	2.0	0.5	0.38	14
Casein, acid hydrolyzed	8.1	4.0	38.5		0.75	14
Casein, enzyme hydrolyzed	12.7	4.1	7.1		1.05	14
Casein, vitamin test	15.5	3.7	3.7	0.2	2.25	7
Casein, enzyme hydrolyzed	12.9				1.35	7
Corn gluten	3.6	12	2	1	0.02	2
Corn (germ) cake meal	3.3	12	2	7	0.03	2
Corn germ, defatted						18
Egg (whole) powder	7.7	5.5	4.5	41		15
Egg (whole) powder	7.6	2.0	3.6	43.8	1.30	3
Egg (whole) powder	6.6	2-5	2.8	38-51	1.45	17
	8.6		5.4			
Fibrin (beef)	13.7	8.0	6.5			3
Fish, meal and solubles	5.1	47.4	12.4	2.2		5
Fish, sardine meal					2.75	6
Gelatin	15.8	9-11	0.3	trace	0.86-1.01	17
Gelatine, porkskin (acid)			0.3		1.50	9
Gelatine, bone ossein (limed)			1.2		1.50	9
Gelatine, calfskin (limed)			1.2		1.50	9
Haemoglobin (beef)	14.0	10	2.2			3
Lactalbumin	11.3	5.5	3.4	8.9		3
Lactalbumin	11.5	5.0	3.5	5.0	0.70	17
Lactalbumin	13.0	3.0	1.5	4.0	0.60	19
Lactalbumin, enzyme hydro- lyzed	11.5				1.75	7
Lactalbumin, enzyme hydro- lyzed	12.0	6.0	4.0		1.40	14
Liver, enzyme hydrolyzed	8.6	12.6	12.6	0.87	5.84	10
Milk (skim) powder	5.9	3.0	8.0	1.0	0.10	17
Protein hydrolysate	11.4	3.2	6.8	0.43	1.64	11
Rabbit, coney hair					0.07	16
Rabbit, shredded pelt					0.035	16
Rabbit, pouncing dust					0.032	16
Silk (waste)†		11	1-1.4	0.5-1		4
Silk fibroin	18.4					4
Yeast, dried	7.1	4.5	6.5	5.8	0.36	1
Yeast, enzyme hydrolyzed	11.5		10.0		1.05	1
Yeast, primary	8	4	7	3	0.45	2
Yeast, primary enzyme hy- drolyzed	11.5	4	10	1.5	1.10	2
Wheat germ, defatted	4.0					18
Wheat germ	13.2	12	5	10	0.02-0.08	8
Wheat gluten		6	7	10	0.12-0.17	8

* The authors are indebted to officers of the representative concerns listed below from whom most of the data given in the table was obtained in April, 1947. Additional information may be obtained from these companies. No commercial sources of the following protein materials have come to the writers' attention: hog hair, zein, fish scale. According to D. F. Chichester (Drug Trade News, April 7, 1947, page 51), intact



Glutamic acid	hydrochloride
Glycine	ethyl ester hydrochloride, trioxalatochromiate, 5-nitro-naphthalene-1-sulfonate, and nitranilate (2,5-dihydroxy-3,6-dinitro- <i>p</i> -benzoquinone)
Histidine	dihydrochloride, mercuric chloride and mercuric sulfate complexes, 3,4-dichlorobenzene sulfonate, and nitranilate
Hydroxyproline	reineckate (tetrathiocyanato-diaminochromiate)
Isoleucine	copper salt
Leucine	2-bromotoluene-5-sulfonate and β -naphthalene sulfonate
Lysine	monohydrochloride, dihydrochloride and picrate
Methionine	mercuric chloride and mercuric sulfate complexes
Phenylalanine	2,5-dibromobenzene sulfonate
Proline	rhodanilate (tetrathiocyanato-dianilidochromiate) and copper salt
Serine	<i>p</i> -hydroxyazobenzene- <i>p</i> '-sulfonate
Threonine	copper salt
Tryptophan	mercuric sulfate complex
Valine	copper salt

If more than one amino acid were isolated from a single protein source it is evident that materials might be saved, time economized and costs reduced. An obvious advantage would be that the relative amounts of the amino acids in a mixture would increase proportionately as other amino acids were removed. Applying this principle to silk, removal of the four most abundant amino acids (alanine, glycine, serine and tyrosine) making up about 80% of this protein would effect about a five-fold increase in the remaining amino acids. Since the residual material would contain about 15% of aspartic acid, and approximately the same percentages of proline and valine, the isolation of these amino acids should be greatly facili-



proteins and protein hydrolysates are offered, or are under development, by some forty firms.

† Brain, pituitary, duodenum, ovary, thyroid, tongue, spinal cord, blood, spleen, heart, lung, adrenal, liver, pancreas, kidney and stomach.

‡ Contains 72.81% fibroin and 19.28% sericin.

Names and Addresses of Concerns Offering Protein Materials

1. American Home Products Co., 22 East 40th St., New York City.
2. Anheuser-Busch, St. Louis, Missouri.
3. Armour and Co., Chicago, Illinois.
4. Cheney Brothers, Manchester, Connecticut.
5. Dehydrating Process Co., 10 High St., Boston, Massachusetts.
6. James Farrell and Co., 260 California St., San Francisco, California.
7. General Biochemicals, Chagrin Falls, Ohio.
8. General Mills, Minneapolis, Minnesota.
9. Charles B. Knox Gelatine Co., Camden, New Jersey.
10. Lederle Laboratories, Pearl River, New York.
11. McKay-Davis Co., 333 West Woodruff Ave., Toledo, Ohio.
12. Mead Johnson and Co., Evansville, Indiana.
13. Packing Products Co., 271 Church St., New York City.
14. Sheffield Farms, 524 West 57th St., New York City.
15. Stein, Hall and Co., 285 Madison Ave., New York City.
16. John B. Stetson Co., Philadelphia, Pennsylvania.
17. Swift and Co., Chicago, Illinois.
18. VioBin Co., Monticello, Illinois.
19. Western Condensing Co., 593 Market St., San Francisco, California.

TABLE II
PERCENTAGES OF AMINO ACIDS IN PROTEINS OF SOME
COMMON NATURAL MATERIALS¹

Amino Acid	Beef Muscle	Blood Powder	Casein	Corn Germ	Corn Gluten
	per cent	per cent	per cent	per cent	per cent
Alanine		4.2 (242)	5.6 (48)		3.1 (49)
Arginine	6.0 (147)	8.1 (48)	4.2 (49)	5.6*	
Aspartic acid		1.8 (48)	6.1 (241)		1.1 (49)
Cystine ²	1.0 (38)	5.7 (48)	0.3 (49)	15 *	24.5 (48)
Glutamic acid	15.2 (147)	trace (48)	22.5 (80)	5.5*	4.3 (49)
Glycine		5.6 (242)	1.9 (230)	3.0*	1.6 (49)
Histidine	3.5 (147)	0 (48)	3.1 (83)		
Hydroxyproline		1.1 (242)	2 (38)		5.0 (49)
Isoleucine	5.3 (147)	12.2 (242)	6.5 (49)	3.8*	24.0 (49)
Leucine	7.7 (147)	8.8 (242)	9.9 (49)	7.1*	0.8 (49)
Lysine	7.9 (147)	1.1 (242)	8.3 (82)	5.3*	2.5 (49)
Methionine	2.5 (183)	7.3 (242)	3.0 (81)	1.5*	6.4 (49)
Phenylalanine	3.9 (147)	2 (38)	5.0 (86)	3.3*	
Proline		5.2 (38)	8.2 (38)		
Serine	5.7 (38)	4.1 (242)	6.4 (241)		4.1 (49)
Threonine	5.4 (147)	1.3 (242)	4.3 (87)	4.5*	0.7 (49)
Tryptophan	0.7 (48)	2.2 (136)	1.4 (219)	1.1 (153)	6.7 (49)
Tyrosine	4.1 (147)	7.7 (242)	5.6 (136)	5.1 (136)	5.0 (49)
Valine	5.2 (147)		6.7 (49)	5.3*	

Amino Acid	Egg powder	Fish scale	Fish muscle	Gelatin	Hair	Lactalbumin
	per cent	per cent	per cent	per cent	per cent	per cent
Alanine		15 (41)	7 (48)	8.3 (132)		0-1 (38)
Arginine	5.6*	6.0*	5.9*	8.0 (147)	9 *	3.3*
Aspartic acid	8.1*	5.3 *	10.6*	5.7 (241)	3.5 (38)	
Cystine ²	2.4 (49)	[0.1] ³ (41)	1.2 (49)	0.1 (48)	16 (48)	4.1 (49)
Glutamic acid	12 *	7.2*	10.1*	11.5 (147)	15 *	18 *
Glycine	3.2*	18.7 *	5.0*	26 (191)	3.6*	2.6*
Histidine	2.4*	0.61*	3.4*	0.79 (147)	1.2*	2.3*
Hydroxyproline		[3] (41)		13 (48)		
Isoleucine	6.7*	0.83*	5.9*	1.4 (147)	3.8*	7.4*
Leucine	9.2*	2.0 *	8.0*	3.2 (147)	6.8*	13.3*
Lysine	7.3*	2.7 *	7.2*	4.1 (147)	3.1*	10.5*
Methionine	3.1*	1.5 *	2.6*	0.76 (154)	0.52*	2.3*
Phenylalanine	5.2*	1.7 *	5.3*	2.2 (147)	2.3*	4.0*
Proline		[12] (41)	3 (48)	15 (132)	4.3 (39)	
Serine		[4.1] (41)	4.5 (49)	3.8 (241)	8.0 (40)	
Threonine	5.3*	1.6 *	4.5*	1.9 (147)	8.2*	6.1*
Tryptophan	1.5 (49)	0.14*	1.3 (49)	0.0 (48)	1.3 (48)	2.1*
Tyrosine	4.5 (49)	[0] (41)	4.4 (49)	0.41 (136)	3.1 (48)	4.4 (49)
Valine	7.0*	1.4 *	5.9*	2.5 (147)	6.0*	6.9*

Amino acid	Milk powder	Silk	Wheat germ	Wheat gluten	Yeast	Zein
	per cent	per cent	per cent	per cent	per cent	per cent
Alanine		26 (33)		5 (48)		9.9 (48)
Arginine	2.8*	1.1 (242)	5.4*	3.9 (49)	5.8*	1.6 (49)
Aspartic acid		2.9 (5)		10 (48)	10.2*	3.4 (48)
Cystine ²	1.2 (48)		0.8 (49)	1.9 (49)	1.3 (48)	1.0 (49)
Glutamic acid	21 *	2.0 (80)	13 *	27 (48)	19 *	23.6 (202)
Glycine	2.3*	44 (33)	5.7*	7.2 (49)	3.9*	0 (49)
Histidine	2.8*	0.34 (83)	2.1*	2.2 (49)	1.5*	1.0 (49)
Hydroxyproline						0.8 (129)
Isoleucine	6.8*	1.1 (242)	3.3*	3.7 (49)	5.5*	5.0 (49)
Leucine	10.0*	0.93 (242)	6.0*	7.5 (49)	6.2*	25.0 (49)
Lysine	8.3*	0.6 (82)	5.1*	2.0 (49)	5.2*	0 (49)
Methionine	2.1*	0.14 (81)	1.2*	1.9 (215)	1.2*	1.7 (175)
Phenylalanine	5.1*	1.3 (86)	2.5*	5.5 (49)	3.4*	6.6 (49)
Proline		3.2 (4)		10 (48)		9-12 (48)
Serine		14.5 (241)				1.0 (206)
Threonine	4.8*	1.2 (87)	3.3*	2.7 (49)	4.0*	2.5 (49)
Tryptophan	2.0 (153)	0.44 (242)	1.0 (49)	0.93 (135)	1.4*	0.1 (48)
Tyrosine	5.0 (136)	13.2 (33)	3.8 (49)	3.8 (49)	2.9 (136)	5.9 (49)
Valine	7.2*	3.5 (242)	4.4*	4.2 (49)	4.2*	3.0 (49)

* Unpublished value obtained in authors' laboratory. The numbers in parentheses are bibliographic citations. The best potential sources of the amino acids are represented by the italic values.

¹ Values calculated to 16% nitrogen.

² Sheep wool, pig hair, and rabbit fur are good sources of cystine but, on the whole, they are less satisfactory materials than human hair. The cost, availability, presence of wool fat (lanolin) or excess pigments (pig hair) and cystine content are some of the critical factors. Cow hair is unsatisfactory since cystine is destroyed by the lime employed in the dehairing process. Horns, hoofs, quills, feathers, egg shell membranes contain relatively low percentages of cystine and, in some instances, troublesome impurities.

³ The percentage values given in brackets are assumed to be the same as those found for fish gelatin (41).

TABLE III
SOME COMMERCIAL SOURCES OF NATURAL (L) AMINO ACIDS*

Amino Acid	Commercial Concern Number																			Price Range per Gram
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
Alanine		x				x	x		x		x			x	x	x		x		\$6.50
Arginine · HCl		x					x					x		x	x	x				0.13 -1.00
Asparagine		x		x			x							x	x	x				0.08 -1.25
Aspartic acid																				0.20
Cysteine · HCl		x				x	x		x		x			x	x	x		x		0.05 -0.22
Cystine	x	x		x		x	x		x		x				x	x		x		0.02 -0.15
3,5-Diiodotyrosine		x																		0.056-0.21
Glutamic acid · HCl						x				x					x	x				0.005-0.01
Glutamic acid						x				x					x	x				0.003-0.10
Glycine		x				x		x		x					x	x				0.005-0.02
Histidine · HCl · H ₂ O		x				x	x			x					x	x		x		0.16 -0.55
Hydroxyproline		x				x	x			x				x	x	x				1.50 -4.50
Leucine		x				x				x					x	x		x		0.005-0.85
Lysine · HCl		x				x	x			x				x		x				0.16 -2.50
Methionine		x																		8.00
Phenylalanine		x							x											2.30 -12.50
Proline		x							x					x	x					0.42 -2.50
Serine		x					x													7.20
Tryptophan		x				x	x		x					x	x			x		1.00 -4.00
Tyrosine		x		x		x	x	x	x		x		x	x	x	x			x	0.007-0.25

* The authors are indebted to officers of the representative concerns listed below from whom most of the prices given in the table were obtained in April, 1947. Additional information on quantities and specifications of the available amino acids may be obtained from these companies. Amino Acid Manufacturers (University of California at Los Angeles) is a source of C.P. and A.P. grades of some of the naturally occurring amino acids.

Names and Addresses of Concerns Offering Natural Amino Acids

1. Becco Sales Company, 420 Lexington Ave., New York City.
2. Bios Laboratories, 607 West 23rd St., New York City.
3. Corn Products Sales Co., 17 Battery Pl., New York City.
4. Digestive Ferments Co., Henry St., Detroit, Michigan.
5. Dow Chemical Co., Midland, Michigan.
6. Eastman Kodak Co., Rochester, New York.
7. General Biochemicals, Chagrin Falls, Ohio.
8. Huron Milling Co., Harbor Beach, Michigan.
9. Interchemical Corp., 1120 Commerce Ave., Union, New Jersey.
10. International Minerals and Chemical Corp., 20 North Wacker Dr., Chicago.
11. Mann Fine Chemicals, 136 Liberty St., New York City.
12. Mearl Corp., 153 Waverly Place, New York City.
13. Merck and Co., Rahway, New Jersey.
14. Myristine Chemical Co., 21 Lafayette Pl., Arlington, New Jersey.
15. Paragon Testing Laboratories, Orange, New Jersey.
16. Planstichl Chemical Co., Waukegan, Illinois.
17. Sidley Chemical Works, 961 Frelinghuysen Ave., Newark, New Jersey.
18. Van Camp Laboratories, Terminal Island, California.
19. Winthrop Chemical Co., Rensselaer, New York.

tated. If these three amino acids were removed, the proportions of the remaining amino acids would be approximately doubled.

Experimental procedures have been reported, especially by early workers in this field, for the isolation of as many as twelve amino acids from a single protein source but in most cases the yields and purity of the amino acids isolated were not entirely satisfactory. Methods of this kind which have been most widely used by previous investigators are discussed below.

1. Ester Method (Fischer)

The isolation of glutamic acid as the hydrochloride and of glycine as the ethyl ester hydrochloride from acid hydrolysates of casein was reported by Fischer (108) in 1901. In addition, other amino acids were separated by fractional distillation of their ethyl esters at 40–160° and 9–10 mm. and leucine, valine, proline, aspartic acid and phenylalanine were isolated as the free amino acids or their salts (copper and barium). Fischer and Skita (118) isolated tyrosine, glycine, alanine, leucine and phenylalanine from acid hydrolysates of silk fibroin by analogous procedures.

2. Silver Salt Method (Kossel-Kutscher)

The slightly soluble silver salts of arginine and histidine were precipitated from strongly alkaline solutions of protein hydrolysates, histidine was separated from arginine by precipitating its silver salt at about pH 7.5 and the basic amino acids were isolated as histidine dihydrochloride, lysine phosphotungstate and picrate and arginine nitrate, picrolonate and copper nitrate double salt. This procedure, described by Kossel and Kutscher (172) in 1900, has been utilized widely for analytical, but not preparative purposes.

3. Butanol Extraction Method (Dakin)

In 1920, Dakin (71) separated amino acids into groups by extracting acid-free solutions of acid-hydrolyzed gelatin with butanol. Proline and the neutral amino acids (except glycine) were readily extracted with butanol while the basic amino acids (arginine, histidine, and lysine) and the acidic amino acids (aspartic acid and glutamic acid) remained behind. Glycine, leucine, phenylalanine, proline, serine, tyrosine, hydroxyproline, aspartic acid, glutamic acid, histidine, arginine and lysine were isolated essentially by adaptations of the fractional distillation and crystallization procedures of Fischer and Kossel. The isolation of alanine, leucine, tyrosine, proline, phenylalanine, aspartic acid and glutamic acid

from zein was reported by Dakin (72) in 1923. Dakin (73) has stated, recently, that the identity of the β -hydroxyglutamic acid isolated from zein and other proteins is uncertain.

4. Copper Salt Method (Town)

Town (256) has separated the amino acids in protein hydrolysates by fractionally crystallizing their copper salts. The fractions and the amino acids in each fraction were (I) copper salts insoluble in water and methanol (cystine, leucine, phenylalanine and tyrosine), (II) copper salts soluble in water and methanol (hydroxyproline, isoleucine, proline and valine) and (III) copper salts soluble in water but insoluble in methanol (alanine, arginine, glutamic acid, glycine, histidine, lysine and serine). An analogous procedure has been employed by Brazier (53) who isolated the amino acids from the copper salt fractions as arginine flavianate, proline picrate and aspartic acid and glutamic acid as the barium salts by the procedure of Foreman (123). Phenylalanine, histidine and leucine were isolated as the zinc salts while alanine, tyrosine and valine were obtained as the free amino acids. The presence of hydroxyvaline was reported both by Brazier (53) and Town (257).

5. Barium Carbamate Method (Kingston and Schryver)

Aspartic acid, glycine, proline, leucine and a mixture of amino acids considered to contain leucine, alanine, phenylalanine, serine and hydroxyproline were obtained from an acid hydrolysate of gelatin by Kingston and Schryver (164) who separated these amino acids as their barium carbamate derivatives.

6. Electrodialysis (Cox, King and Berg)

Leucine and tyrosine were isolated by Cox, King and Berg (69) from an acid-free sulfuric acid hydrolysate of blood corpuscle paste. The filtrate was electrodialyzed in a three-compartment apparatus and the basic amino acids were isolated from the catholyte as arginine flavianate, histidine mercuric sulfate complex and dihydrochloride and lysine picrate.

7. Ionophoresis (Consden *et al.*)

Consden, Gordon and Martin (65) have separated the amino acids of wool hydrolysates into acidic, neutral and basic fractions by ionophoresis in silica gel. The amino acids in mixtures containing glycine and serine, alanine and methionine, lysine and histidine, or glutamic acid and aspartic acid were separated by this method.

8. Anhydrous Fatty Acid Method (von Przylecki and Kasprzyk)

The amino acids have been separated by von Przylecki and Kasprzyk (210) into the three groups a) soluble in acetic or other anhydrous fatty acid (basic amino acids), b) soluble in a mixture of an anhydrous fatty acid and 0.2 to 5% water (all neutral amino acids except cystine and tyrosine), and c) insoluble under either set of conditions (aspartic acid, glutamic acid, cystine and tyrosine).

9. Aromatic Sulfonic Acid Method (Stein *et al.*)

Tyrosine has been crystallized directly by Stein *et al.* (237, 238) from the acid-free hydrolysate of silk fibroin and other amino acids have been isolated as the following aromatic sulfonates: glycine, 5-nitronaphthalene-1-sulfonate, alanine, azobenzene-*p*-sulfonate and serine, *p*-hydroxy-*p'*-sulfonate. Leucine, 2-bromotoluene-5-sulfonate and phenylalanine, 2,5-dibromobenzene sulfonate were obtained from the hydrolysate of technical hemoglobin.

10. Chromatography*

Almost all of the amino acids have been separated by chromatography, a process first described by Tswett (259) in 1906. The types of adsorbents and ion exchange materials employed and the underlying principles have been discussed by Wieland (278), Tiselius (249-251), Cannan (55), Meyers (188), Martin and Synge (187), Zechmeister and Chohnoky (284), Strain (243) and Cawley (57). Examples of adsorbents used are cellulose (64), starch (102, 236, 247), activated carbon (167, 248, 276), silica gel (132, 133), and silver sulfide (138, 139). The ion exchangers employed include aluminum oxide (75, 278), natural and synthetic zeolites (Fuller's earth [260, 278]), Permutite (220, 281), Lloyd's Reagent (26), Hyflo Super Cel (26) and synthetic resins containing functional amino and sulfonic acid groups (De-Acidite, Duolite, Wofatite and Amberlite (62, 104, 261, 278, 279)). The specificity and completeness of adsorption or exchange of amino acid anions and cations depend upon the type and particle size of the adsorbent or exchanger, length of the adsorbing column, rate of flow of the solution, concentration of amino acids in the solution, pH of the solution, and other factors. Most of the investigations reported have been concerned, primarily, with the governing principles of adsorption and ion exchange of mixtures of purified amino acids.

* Excellent reviews of this topic have been presented by Cassidy (twelve collaborating authors) (56a), Strain (243a), Clegg (63a), and Kirk and Duggan (164b).

Block (44-47) has employed cation exchange resins (Amberlite IR-100) for the separation of the basic amino acids and anion exchange resins (Amberlite IR-4) for the separation of the dicarboxylic amino acids. The basic amino acids were precipitated as their silver salts by a modified Kossel-Kutscher procedure and these amino acids were isolated as arginine flavianate, histidine nitraniolate and lysine picrate. The yields of these amino acids obtained from an acid hydrolysate of commercial blood fibrin were 6.4%

TABLE IV
RESOLUTION OF AMINO ACIDS

Amino Acid	Derivative	Alkaloid or Derivative for		Reference Number
		L-amino Acid	D-amino Acid	
Alanine	benzoyl	brucine	strychnine	105
	benzoyl	strychnine	brucine	88, 105, 110
	<i>m</i> -nitrobenzoyl	quinine	brucine	63
Aspartic acid	benzoyl	brucine	brucine	105
Cystine	acetyl	brucine	brucine	275
	formyl	strychnine	strychnine	275
	formyl	brucine	brucine	180
Glutamic acid	benzoyl	strychnine	strychnine	105
Histidine	—	tartaric acid	tartaric acid	211
Hydroxyproline	phenylisocyanate	quinine	quinine	176
Isoleucine	formyl	brucine	brucine	11
Leucine	benzoyl	quinidine	cinchonine	107
	formyl	brucine	brucine	120
Lysine	—	d-camphoric acid	l-camphoric acid	255
Methionine ✓	formyl	brucine	brucine	156
Phenylalanine	formyl	brucine	brucine	117
	benzoyl	cinchonine	cinchonine	116
Proline	<i>m</i> -nitrobenzoyl	cinchonine	cinchonine	121
Serine	<i>p</i> -nitrobenzoyl	brucine	quinine	115
Threonine	formyl O-methyl	brucine	brucine	280
Tryptophan	acetyl	quinine	quinine	25
Tyrosine	benzoyl	brucine	cinchonine	106
	formyl	brucine	brucine	6
	acetyl	—	brucine	229a
Valine	formyl	brucine	brucine	111

arginine, 2.0% histidine and 7.5% lysine. About 98-100% of the lysine, 75% of the histidine and 50% of the arginine in protein hydrolysates were present in the effluent obtained by large-scale operation.

11. Chemical Resolution

Both optical antipodes of the amino acids have been prepared by resolution of the DL-forms. The salts formed with optically active bases such as quinine, strychnine and brucine and the formyl, benzoyl and other derivatives of the D- and L-amino acids in the

DL-mixtures are separated by fractional crystallization (85, 113). The general principles governing resolutions have been discussed by Shriner *et al.* (231). The spontaneous resolution of DL-histidine and isolation of the histidine antipodes have been reported by Duschinsky (89). Alkaloids and amino acid derivatives commonly employed in the resolution of the amino acids are shown in Table IV.

12. Enzymatic Oxidation

One antipode of a DL-amino acid is oxidized more rapidly than the other to the α -keto acid and other products by the action of a mold, yeast, bacterium or amino acid oxidase. Either the D- or the L-isomer may be isolated from the reaction products depending upon the characteristics of the organism or the oxidase employed. Duschinsky and Jeannerat (90) and Behrens (23) have prepared the L-antipodes of alanine, isoleucine, methionine, and valine by oxidizing the D-form of the DL-mixtures with D-amino acid oxidase obtained from hog kidney. The literature on this topic prior to 1922 has been reviewed by Ehrlich (96). The organisms and amino acid oxidases employed and the amino acid antipodes isolated are listed in Table V.

13. Enzymatic Resolution

The DL-amino acids have been resolved by Bergmann *et al.* (28, 29, 37, 128) by selective enzymatic synthesis. If an acyl derivative of a DL-amino acid is incubated with aniline or phenylhydrazine in the presence of a suitable enzyme under favorable proteolytic conditions, the anilides or phenylhydrazides of the antipodes are formed. The derivative of the L-isomer is obtained as a crystalline precipitate while that of the D-isomer is precipitated as an oil by acidifying the filtrate. Carbobenzoxy derivatives are used for this purpose since the carbobenzoxy radical may be removed as toluene and carbon dioxide by catalytic hydrogenation at room temperature. Anilides and hydrazones of amino acid antipodes have been prepared with the aid of cysteine- and HCN-activated papain, bromelin (from pineapple) and cathepsin (from pig liver) but only D- and L-leucine and D- and L-glutamic acid have been obtained by this procedure. The L- and D-antipodes of sixteen amino acids have been prepared recently by Greenstein *et al.* (121a, 121b, 130a, 135a) who subjected N-chloroacetyl derivatives of the DL-forms to asymmetric hydrolysis by an enzyme preparation from hog kidney or carboxypeptidase from beef pancreas.

TABLE V
ENZYMATIC OXIDATION OF AMINO ACIDS

Amino Acid Isolated	Oxidizing System	Reference Number
D-Alanine	yeast	93
L-Alanine	takadiastase	152, 199
L-Alanine	D-amino acid oxidase (kidney)	23, 90
D-Arginine	liver	216
D-Glutamic acid	mold	209, 227
D-Glutamic acid	yeast	95
D-Histidine	yeast	10, 95
D-Histidine	dog	10, 165
D-Isoleucine	yeast	95
D- and L-Leucine	pancreatin	277
L-Leucine	<i>Penicillium glaucum</i>	227, 228
D-Leucine	L-amino acid oxidase (<i>Proteus vulgaris</i>)	244
D-Methionine	L-amino acid oxidase (<i>Proteus vulgaris</i>)	244
L-Methionine	D-amino acid oxidase	90
D-Phenylalanine	yeast	94
D-Phenylalanine	L-amino acid oxidase (<i>Proteus vulgaris</i>)	244
L-Proline	D-amino acid oxidase (kidney)	239
D-Serine	yeast	94
D-Tyrosine	pancreas	7
D-Tyrosine	yeast	97, 98
D-Tyrosine	<i>Oidium lactis</i>	60, 98
D-Valine	yeast	99

III. PROTEIN HYDROLYSIS

In 1820 Braconnot (52) hydrolyzed gelatin by boiling this protein with dilute sulfuric acid. Since that date proteins have been hydrolyzed by heating them for times up to 48 hours and temperatures up to 180° with hydrochloric acid or sulfuric acid (and some other acids) at concentrations as high as about 16 *N*. Hydrolysis has also been effected by heating proteins with barium hydroxide solutions or other alkalies and by incubating them with pancreatic and other proteolytic enzymes. The object has been to effect complete liberation of the amino acids and to avoid any degradation or change in the amino acid forms occurring in the native proteins.

It was observed by early workers that varying amounts of brown or black decomposition products (soluble humin) were formed during acid hydrolysis of most proteins, that a black precipitate (insoluble humin) usually formed and that the quantity of humin was roughly proportional to the impurities (mainly carbohydrates) in the protein material. Since, under such circumstances, nearly all of the tryptophan and large but varying amounts of cystine and tyrosine were found to be destroyed, acid hydrolysis has been carried out in the presence of stannous chloride (149) and other reducing agents, or in an atmosphere of nitrogen, to prevent or

TABLE VI

SUMMARY OF PROTEIN HYDROLYSIS CONDITIONS EMPLOYED IN AMINO ACID ISOLATION PROCEDURES DESCRIBED IN SECTION IV

Amino Acid	Protein	Acid			Hydrolysis Time (Hours) ^b	Reference Number
		Type	Normality	gm./gm. Protein		
Alanine	gelatin	HCl	11.7	6.0	6	177
	glutenin	H ₂ SO ₄	13.7	4.0	24	74
	silk fibroin	HCl	11.7	4.8	12	33
	degummed silk	HCl	11.7	3.4	8	238
	silk fibroin	HCl	11.7	4.8	5	118
Arginine	gelatin	HCl	11.7	2.7	24	66
Aspartic acid	casein	HCl	11.7	3.6	6	108
	casein	HCl	9.8	3.5	48	123
Cystine	human hair	HCl	8.0	3.4	8	193
Glutamic acid	wheat gluten	HCl	9.9	3.5	6	16
	gliadin	HCl	6.2	2.2	48	205
	zein	H ₂ SO ₄	11.6	3.8	28	72
Glycine	protein ^a	HCl	11.7	3.0	6	110
Histidine	blood	HCl	11.7	3.8	20	125
	hemoglobin	HCl	11.7		8	266
Hydroxyproline	gelatin	H ₂ SO ₄	12.0	6.0	°	158
	gelatin	HCl	11.7		8	27
Isoleucine	cod muscle	HCl	6.9	11.1	40	51
	fibrin	Papain	°	7.8	20 ^d	32
	silk	HCl	11.7	3.6	6	254
Leucine	hemoglobin	H ₂ SO ₄	13.7	3.8		238
Lysine	blood	H ₂ SO ₄	14.8	4.9	30	69
	blood	H ₂ SO ₄	15.8	5.6	24	27
Phenylalanine	hemoglobin	H ₂ SO ₄	13.7	3.8		238
Proline	gelatin	H ₂ SO ₄	11.7		8	27
Serine	degummed silk	HCl	11.7	3.4	8	238
	silk fibroin	H ₂ SO ₄	9.8	6.8	18	214
Threonine	blood fibrin	H ₂ SO ₄	13.0	5.9	16	119
Tryptophan	casein	pancreatin	f	10.0	12 ^e	198
	casein	pancreatin	f	10.0	12 ^e	198
Tyrosine	blood	H ₂ SO ₄	14.8	4.9	30	69
	horn	HCl	11.7	4.0	10	68

^a General method applied subsequently to various proteins.^b Signifies, unless otherwise indicated, reflux time at atmospheric pressure.^c Autoclave conditions.^d 20 days at 37°.^e 0.29% papain in solution maintained at pH 6.4 with buffers.^f 0.7% pancreatin in solution made alkaline with excess sodium carbonate.^g 12 days at 37°.

minimize decomposition. Numerous studies have been made in an attempt to establish optimal hydrolytic conditions but the results obtained appear uncertain owing in part to the unreliability of some of the methods employed for the determination of the resulting amino acids. Hydrolysis of proteins with alkali has been found unsatisfactory because cystine, arginine, tyrosine and some other amino acids are wholly or partly destroyed and all amino acids are completely or partly racemized depending upon the time and temperature of hydrolysis, the concentration of the alkali and other factors. The hydrolysis of proteins by enzymes requires considerable time and is usually incomplete.

Since it has not been found possible to establish ideal hydrolytic conditions most investigators have adopted arbitrary conditions such as refluxing mixtures of proteins with 6 *N* HCl for 24 hours and incubating mixtures of proteins and pancreatin for several weeks

at 37°. This topic has been discussed at length by Mitchell and Hamilton (190), Calvery (54) and Block and Bolling (48). A summary is given in Table VI of the hydrolysis conditions employed by the investigators whose isolation procedures have been described in some detail in Section IV.

IV. ISOLATION PROCEDURES

The procedures which are considered to be the most practicable for the isolation of the common amino acids are outlined below:

1. Alanine

The best natural source of alanine is silk which contains nearly 25% of this amino acid. Other proteins contain less than 10% of alanine. Alanine may be readily isolated by the classical ester procedure of Fischer (108) which has been applied to silk, silk fibroin, sericin, casein, gelatin and other proteins. Glutamic acid hydrochloride is crystallized from the protein hydrolysate, the remaining amino acids are esterified and glycine ethyl ester hydrochloride is allowed to crystallize. The free amino acid esters are fractionally distilled *in vacuo* and alanine is obtained from the ethyl ester fraction, b.p. about 50° at 10 mm. The yield of high purity alanine from silk fibroin is about 20% (118).

A satisfactory isolation procedure has been described recently by Stein *et al.* (236a, 238). Silk fibroin is refluxed with concentrated hydrochloric acid, the bulk of the acid is removed by vacuum distillation and treatment with lead acetate, tyrosine is precipitated and the suspension filtered, and glycine is precipitated from the filtrate as the 5-nitronaphthalene-1-sulfonate. The suspension is filtered, methyl cellosolve is added, alanine is precipitated as the azobenzene sulfonate,* the azobenzene sulfonic acid is removed as the barium salt, and the alanine obtained from the filtrate is recrystallized from aqueous ethanol. The yield of high purity alanine from technically degummed silk is about 22%.

By the method of Bergmann and Niemann (33) glycine is removed from an acid hydrolysate of silk fibroin as its trioxalatochromiate and alanine is precipitated as its dioxypyridate. The latter is obtained in a yield equivalent to about 25% of alanine and it seems probable that the alanine could be recovered satisfactorily

* See Stein and Moore (236b) for preparation of azobenzene-*p*-sulfonic acid trihydrate.

from the dioxypyridate. Disadvantages of this and other comparable procedures are the cost of the sulfonic acid reagents and the time required to prepare them.

Behrens (23) prepared 22 grams of L-alanine by an enzymatic oxidation procedure. Oxygen was passed for five hours with vigorous stirring into a solution containing 72 grams of DL-alanine and an aqueous extract of dry, powdered pig kidney (120 grams). The solution was acidified, heated to boiling and filtered. The filtrate was concentrated *in vacuo*, trichloroacetic acid was added, the suspension was filtered and the filtrate was evaporated to dryness *in vacuo*. A solution of the residue containing L-alanine hydrochloride was treated with aniline and the resulting crude L-alanine was recrystallized from aqueous ethanol.

2. Arginine

Arginine is readily isolated from gelatin as the monohydrochloride by Cox's (66) modification of Kossel and Gross's (171) method. The yield is about 6% from purified gelatin but it is somewhat less from ground-hide glue (low grade gelatin). Gelatin is hydrolyzed with HCl, the excess acid is distilled *in vacuo*, arginine is precipitated as the monoflavianate, the flavianic acid is removed, aniline is added to the filtrate and the resulting arginine monohydrochloride is purified by treatment with norit and recrystallization from aqueous-ethanol. A mixture of the mono- and di-flavianates may crystallize unless arginine flavianate is precipitated initially at about pH 4. Other modifications of the Kossel procedure, including preliminary electrodialysis (69) have been described for the isolation of arginine from gelatin, hemoglobin (31, 69, 269), human hair (270), fibrin (30), protamines (169, 170), salmon muscle (17) and other proteins. Arginine has been isolated as other types of salts including the silver salt, the picrolonate, the silver nitrate salt, the nitrate and the benzylidene derivative. Free arginine may be prepared by treating a solution of the monohydrochloride with silver oxide, filtering the suspension of silver chloride, and evaporating the filtrate *in vacuo* in a stream of carbon dioxide-free air (127).

According to the present authors' experience, human hair hydrolysates from which the cystine has been removed and low-grade gelatin are satisfactory sources of arginine. C. P. HCl should be used in hydrolyzing the proteins and the arginine flavianate should

be recrystallized before converting it to the monohydrochloride. Arginine flavianate has been recrystallized from dilute ammonia by Kossel and Gross (171). It may be purified satisfactorily by dissolving the crude product in eight volumes of 0.2 *N* NaOH, decolorizing the solution with a quantity of norit A equivalent to 10% of the weight of the flavianate and adding 6 *N* HCl to pH 3 (blue color of Congo red indicator).

Arginine flavianate may be decomposed by hydrolyzing it with HCl. The suspension of flavianic acid is filtered and traces of flavianic acid in the filtrate may be removed by distilling the excess HCl *in vacuo* and diluting the residual solution with water to precipitate the slightly soluble arginine flavianate. The pale yellow filtrate is decolorized with norit A, the clear filtrate is evaporated to dryness *in vacuo*, the residue is taken up in hot 95% ethanol and the arginine monohydrochloride is precipitated by the addition of concentrated ammonium hydroxide and seeds of pure arginine monohydrochloride. The base should be added slowly to prevent oil formation and any oil that separates should be allowed to crystallize before adding more base. Pyridine, monoethanolamine and other organic bases are considered to be more satisfactory than aniline for this purpose. Block (46) has employed an anion exchange resin (Amberlite IR-4), instead of HCl, for the decomposition of arginine flavianate.

The purification of arginine as the benzylidene derivative has been described by Bergmann and Zervas (35, 36) but this step does not appear to be an essential one. Block (44) has isolated 6.4% of arginine (as flavianate) from blood fibrin hydrolysate after adsorbing traces of HCl with Amberlite IR-4 and the basic amino acids with Amberlite IR-100, eluting the basic amino acids from the column with HCl, and precipitating the histidine and arginine as the silver salts by the Kossel procedure.

3. Aspartic Acid

Aspartic acid is isolated most conveniently from asparagine by Vickery and Pucher's (271) modification of Schiff's (222) method. Asparagine monohydrate is hydrolyzed with HCl, the solution is adjusted to pH 3, two volumes of 95% ethanol are added and the aspartic acid precipitate is recrystallized from boiling water.

Asparagine is readily isolated from the etiolated seedlings of *Lupinus albus* (272) and *Lupinus angustifolius* (271) by the methods

of Vickery and coworkers. It has been found in the writers' laboratory that the seedlings may be grown satisfactorily in moist sand in a light-tight cabinet or in soil in light-tight beds. Fungus contamination was practically eliminated by regulating the moisture content of the substrates at the minimum level required for germination and satisfactory growth of the seedlings. The weight of the moist seedlings grown for 12 days is about 10 times that of the seeds. The moist seedlings (sprouts, roots and testa) are ground or macerated (a Waring blender is convenient) and the mass is filtered, pressed or centrifuged in a basket centrifuge. The combined filtrates and press juice are heated to coagulate the protein, acetic acid is added to pH 6, the suspension is filtered on a Celite pad, the filtrate is concentrated *in vacuo*, and the syrup is seeded with crystals of asparagine monohydrate. The crude asparagine monohydrate is recrystallized from about five volumes of distilled water. The yield of purified asparagine monohydrate is about 10% of the dry weight of the seeds.

The methods described by Vickery, *et al.* were essentially the same as those employed previously except that early workers usually precipitated proteins and other impurities from the solution with lead acetate. Asparagine was first isolated from asparagus juice by Vacquelin and Robiquet (263) although, according to Winterstein and Huber (283), asparagus sprouts contain only about 0.2% of asparagine. The methods employed and the results obtained by Piria, Schulze and other early workers have been reviewed by Winterstein (282), Vickery and Schmidt (273), and Chibnall (58). More recently, Vickery *et al.* (264, 265, 271, 272) have investigated the amide and asparagine content of alfalfa and the seedlings of the soybean, the purple vetch (*Vicia atropurpurea*), *Vicia villosa*, the broad or horse bean (*Vicia faba*) and the summer squash (*Curcubita pepo*). Asparagine has been isolated from the poppy, sunflower, beet, potato and numerous other plant species listed by Steele (235) and Onslow (204).

Aspartic acid has been isolated from proteins as barium aspartate by Fischer's (1) method, as calcium and copper aspartates by Foreman's (123) modification of Ritthausen's (217) procedure and by modifications or adaptations of these methods described by Chibnall, *et al.* (59) and other workers. It seems probable that adsorption of the acidic amino acids with an anion-exchange resin (Amberlite IR-4, Duolite A-3, or De-Acidite) (65a) or separation of

the acidic amino acids by electrodialysis would be an advantageous preliminary step in the isolation of aspartic acid from protein hydrolysates.

4. Cystine

High-purity cystine is readily obtained from human hair in about 5% yield essentially by Mörner's (193) method. Men's hair freed from foreign materials and extracted with cleaning solvent is hydrolyzed with HCl, the suspension of humin is filtered, the filtrate is decolorized and brought to pH 3.5, and the crude cystine is purified by isoelectric precipitation and thorough washing with hot water to remove traces of tyrosine and other impurities. Essentially this procedure has been used by Folin (122), Gortner and Hoffman (134), Vickery and Leavenworth (270), Lucas and Beveridge (181), and other workers referred to by these authors. Cystine has also been isolated as the phosphotungstate (253), the mercuric sulfate complex (145), *bis* 3,4-dichlorobenzenesulfonate (274), copper salt (252), and cuprous cysteine derivative (181).

Yields of cystine considerably higher than 5% have been reported by some workers but the purity of the products was not established in most cases. Since values as high as 18% of cystine in hair have been found by colorimetric analysis, it would be expected that yields of high-purity cystine higher than 5% could be obtained. According to literature reports (61), hair from different species of animals and from humans of different sexes, ages and races varies widely in cystine content.

5. 3,5-Diiodotyrosine

3,5-Diiodotyrosine was first isolated by Drechsel (78) in 1896 from the alkaline hydrolysate of the horny skeleton of the coral, *Gorgonia cavolinii*. This amino acid has been isolated from gorgonia skeletons (245), bath sponges (13), iodinated proteins (207), thyroid (141, 144), and thyroglobulin (245).

6. Glutamic Acid

Glutamic acid is isolated in the laboratory from wheat gluten by the procedure described by Anslow and King (16) and King (163). Wheat gluten is hydrolyzed with concentrated HCl, the solution is decolorized and the suspension is filtered, the filtrate is concentrated *in vacuo* and the combined first and second crops of glu-

tamic acid hydrochloride are recrystallized from 6 *N* HCl. The yield is about 26%. Yields as high as about 42% have been obtained from gliadin (205). The yield from zein (72) is about 31% and from other proteins less than 20%. Glutamic acid is prepared from the hydrochloride or commercial monosodium glutamate (223) by isoelectric precipitation at pH 3.

Glutamic acid is isolated commercially, usually as the hydrochloride, from wheat gluten and the waste water (Steffen's waste) obtained from the manufacture of beet sugar. Steffen's waste, specific gravity about 1.05 and glutamic acid content about 0.1%, is treated with carbon dioxide to remove lime and is evaporated under reduced pressure to a specific gravity of about 1.4 and glutamic acid content ranging from 1 to 6% depending upon the source of the material. Potassium chloride, betaine hydrochloride and glutamic acid hydrochloride are separated by procedures which are described in numerous patents referred to by Laurence, (174), O'Day and Bartow (201), Benninghoff (24), Albroom (14), Eldridge (100), Barta (21) and Lyon (184). The residual solutions obtained in the manufacture of glutamic acid from wheat gluten and Steffen's waste represent potential sources of tyrosine (22, 186), leucine (130, 186), and some other amino acids. (See Introduction for discussion and references to literature.)

7. Glycine

Gelatin (191) contains about 26% of glycine and silk fibroin (33) about 44%; these proteins are the best sources of this amino acid. By Fischer's (113) ester method, the filtrate from the glutamic acid hydrochloride suspension is evaporated, the syrup is esterified with absolute ethanol and dry HCl gas, the solution is evaporated and allowed to stand at room temperature and the resulting glycine ethyl ester hydrochloride is recrystallized from absolute ethanol. Although no procedure has been reported, glycine probably could be readily prepared by hydrolyzing the ester. Diketopiperazine and glycine polypeptide esters might be formed from glycine ester but glycine would result from the hydrolysis of these products as well as from glycine ester. Glycine has been isolated from numerous proteins as its ethyl ester hydrochloride, copper salt (232), nitroanilate (258), trioxalatochromiate (28, 42), 5-nitronaphthalene-1-sulfonate (192) and picrate (67). Relatively large amounts of glycine are manufactured for clinical and other purposes by the amination of chloroacetic acid.

8. Histidine

Histidine is isolated from hemoglobin by the method of Vickery (266). Technical hemoglobin is hydrolyzed with HCl, the solution is distilled *in vacuo* to remove excess HCl, and histidine *bis* 3,4-dichlorobenzenesulfonate is crystallized. The crude product is recrystallized from five to ten times its weight of boiling distilled water. The smaller ratio is used if the crystals are largely stout prisms (histidine 3,4-dichlorobenzenesulfonate) and the larger ratio if masses of fine needles (leucine 3,4-dichlorobenzenesulfonate) are observed.* The sulfonic acid is removed as its barium salt, the filtrate is concentrated *in vacuo* and the precipitate is recrystallized from 50% ethanol. The yield of purified histidine is about 6% from purified hemoglobin. It has been found that the preparation of histidine is more difficult and that the yields are lower from technical than from purified hemoglobin. The precipitation of histidine sulfonate, while retaining the leucine sulfonate in supersaturated solution, appears to be a critical point. For this reason, one or more recrystallizations of the initial histidine sulfonate may be required to facilitate the purification of the histidine hydrochloride. It might be expected that histidine sulfonate could be isolated more satisfactorily from the catholyte obtained by electrodialysis or the eluate from a cation exchange column than from an untreated protein hydrolysate.

Histidine is isolated from dried blood corpuscle paste by Foster and Shemin's (125) modification of Kossel's (168) procedure. The HCl hydrolysate of corpuscle paste is brought to pH 4.4, the crude precipitate containing tyrosine and leucine is removed, an alcoholic solution of mercuric chloride is added, the solution is brought to pH 7.5, the histidine-mercuric chloride suspension is filtered, the complex is decomposed with H_2S and histidine monohydrochloride is crystallized from the filtrate of the mercuric sulfide suspension. This product is recrystallized from ethanol. The yield is about 6%. The chief disadvantage of this method is the cost of the mercuric chloride and the difficulty of recovering it from the end product, mercuric sulfide.

Histidine has been isolated from various proteins as the mercuric chloride and mercuric sulfate (31) complexes, the monoflavinate (31), the diflavinate (267), the nitroanilate (43) and the picrolonate (17). The use of dioxane, isopropanol and *n*-propanol for the ex-

* Histidine and leucine 3,4-dichlorobenzene-sulfonates may be separated by extracting the latter with acetone (53a).

traction of histidine from protein hydrolysates has been suggested by Gilson (131). Free histidine is prepared by dissolving the mono- or dihydrochloride in water, adding an excess of hot silver sulfate, filtering the suspension of silver chloride and silver sulfate, removing the silver ions from the solution as silver sulfide and adding an equal volume of ethanol to the filtrate. It may be prepared as readily by adding an equivalent quantity of ammonium hydroxide or lithium hydroxide (91) to an aqueous solution of the hydrochloride.

9. Hydroxyproline

Hydroxyproline is isolated in 6.5% yield from gelatin by Neuberger's (200) method. Arginine is removed as the flavianate from an HCl hydrolysate of gelatin, lysine and histidine are removed as the phosphotungstates from the diluted filtrate, excess phosphotungstic acid and flavianic acid are extracted with aqueous ammonia and ether, proline and hydroxyproline are precipitated as their reineckates, the latter are decomposed with CuSO_4 and SO_2 , copper is removed as the sulfide, sulfate is removed as barium sulfate, the filtrate is evaporated to dryness, proline is extracted from the residual material with absolute ethanol and the crude hydroxyproline is recrystallized from 80% ethanol.

Kapfhammer and Eck (158) isolated 7% of hydroxyproline from gelatin. Arginine was removed from the hydrolysate as the flavianate, proline and hydroxyproline were precipitated as the reineckates, the precipitate was decomposed with CuSO_4 and SO_2 , the filtrate was evaporated to dryness, the residual material was extracted with ethanol to remove proline and the crude hydroxyproline was recrystallized from ethanol. Bergmann (27) isolated 10.5% of hydroxyproline as the reineckate from a gelatin hydrolysate after arginine had been removed as the flavianate and proline as the rhodanilate. The hydroxyproline reineckate was decomposed with pyridine. Klabunde (166) isolated 2% of hydroxyproline from gelatin. The copper salts of the amino acids were prepared and extracted with methanol, copper was removed as the sulfide from the methanol-soluble copper salts, the filtrate was evaporated to a sirup, proline was extracted from the latter with absolute ethanol and hydroxyproline was precipitated as the pierate from a solution of the ethanol-insoluble material. The pierate was decomposed with aniline and the crude hydroxyproline was recrystallized from aqueous methanol. Hydroxyproline was first isolated by Fischer (109) from a gelatin hydrolysate after removing glycine as the ethyl

ester hydrochloride and the diamino acids as the phosphotungstates. Methods have been described for the isolation of hydroxyproline as the cadmium chloride complex (159), the platinum and gold chloride complexes (103) and the N-acetyl-O-benzoyl derivative (246). Difficulty has been encountered by the writers and by some other workers in preparing hydroxyproline of high purity in satisfactory yield.

10. Isoleucine

Isoleucine was isolated from de-sugared molasses by Ehrlich (92) in 1904. The less soluble copper salt of leucine was separated by fractional crystallization from the more soluble copper salt of isoleucine. Isoleucine has been isolated from various proteins by this procedure but no highly practicable method for the isolation of isoleucine is available. Isoleucine has been isolated from crude commercial leucine as the *p*-toluenesulfonyl derivative (146).

11. Leucine

Leucine is isolated from hemoglobin by the method of Stein, *et al.* (238). Technical hemoglobin is hydrolyzed with sulfuric acid, the sulfate ions are removed as BaSO_4 , the filtrate is concentrated *in vacuo*, the suspension is filtered and the precipitate is dried. The dry powder is dissolved in boiling water, leucine is precipitated as the 2-bromotoluene-5-sulfonate, the sulfonic acid is removed as the barium salt, and the crude leucine is recrystallized from ammonium hydroxide. The yield of sulfur-free leucine is about 8%.

In 1820, Braconnot (52) hydrolyzed wool with sulfuric acid, added calcium carbonate, evaporated the filtrate and crystallized leucine from the sirup. Essentially this procedure has been used by most workers although it has been common practice to remove tyrosine, glutamic acid, glycine and proline from hydrolysates and to obtain a crude leucine fraction by fractional distillation of the ethyl esters or by fractional crystallization of the copper or other metal salts of the remaining amino acids. A crude precipitate containing leucine, tyrosine, methionine and other amino acids has been obtained at pH 4 in some procedures as a preliminary step in the isolation of the basic amino acids (196). Barnett (20) isolated leucine from HCl hydrolysates of casein and wheat gluten by salting out DL-leucine hydrochloride with NaCl, but, according to Baptist and Robson (18) this product is contaminated with phenylalanine and methionine. Patented processes for the isolation of

leucine from corn gluten have been described by Mark (186) and Gerber (130).

Measures taken to purify the crude leucine include extraction with hot glacial acetic acid to eliminate the slightly soluble tyrosine (137), removal of valine as its water-soluble lead salt (179) and removal of isoleucine as its hot methanol-soluble copper salt (178). It has been found more recently that leucine, free from methionine and apparently other amino acids, is readily prepared by fractionally crystallizing crude leucine as its formyl (126), β -naphthalene-sulfonyl derivative (34, 247a) or monohydrochloride (240).

Hotchkiss (155) has shown that D-leucine is present in gramicidin, an antibiotic substance elaborated by *Bacillus brevis*, by isolating this antipode as its β -naphthalene sulfonate.

12. Lysine

Lysine is isolated as the monohydrochloride from blood corpuscle paste by the method of Rice (214, 214a). A sulfuric acid hydrolysate of blood corpuscle paste is freed from sulfate ions, the suspension of amino acids formed on evaporating the filtrate *in vacuo* is filtered and picric acid is added to the filtrate. The precipitated lysine picrate is recrystallized from water, picric acid is removed by treatment with HCl and extraction with benzene and the lysine dihydrochloride is recrystallized from ethanol-ether. The dihydrochloride is treated with pyridine and the resulting lysine monohydrochloride is recrystallized from aqueous ethanol. The yield of purified lysine monohydrochloride is about 1.2 gm. per 100 grams of blood corpuscle paste.

Lysine is prepared in about the same yield by the method of Cox, King and Berg (69) who separated the arginine and lysine from the histidine by electrodialysis and removed the arginine as the flavianate before crystallizing the lysine picrate. Kossel (169) first isolated lysine as the picrate after removing histidine as the mercuric chloride complex and arginine as the silver salt. By a later procedure (170), Kossel precipitated the basic amino acids as the phosphotungstates according to the method of Drechsel (77) and removed histidine and arginine as their silver salts before precipitating lysine picrate. This method has been widely used primarily for the determination of lysine in proteins. The isolation of lysine as the silver nitrate double salt (77), the dibenzoyl derivative (77), the chloroplatinate (79), the benzylidene and O-hydroxybenzylidene derivatives (35), and ϵ -benzoyllysine copper (173) has been

reported. By the procedure of Block (44) HCl and the dicarboxylic amino acids are removed from an HCl hydrolysate of blood fibrin with an anion-exchange column. The basic amino acids are adsorbed on a cation-exchange column, arginine and histidine are precipitated as their silver salts from the eluate and lysine picrate is precipitated from the filtrate.

13. Methionine

Methionine has been isolated from casein and other proteins by Mueller (194, 195) and other workers (3, 19, 148, 208) who precipitated methionine as a mercuric salt complex. Only small amounts of methionine were obtained and most of the products were somewhat impure. Yields of L-methionine up to about 80% of that (3.5%) assumed to be present in casein have been reported recently by Elvilich-Gomolka (99a). The amino acids adsorbed on activated carbon were eluted with 2 *N* NH₄OH and the L-methionine was purified by fractional crystallization. The purity of the product is questionable since purity was established only by melting point.

14. Phenylalanine

Phenylalanine is isolated from technical hemoglobin in about 1.7% yield by the method of Stein, *et al.* (238). The filtrate from the precipitation of leucine 2-bromotoluene-5-sulfonate is treated with 2,5-dibromo-benzenesulfonic acid, the resulting phenylalanine 2,5-dibromobenzenesulfonate is recrystallized from aqueous methyl cellosolve, the product is dissolved in aqueous pyridine, ethanol is added and the precipitated phenylalanine is recrystallized from a mixture of water, ammonia and ethanol.

Phenylalanine was first isolated as its copper salt from extracts of lupine seedlings by Schulze and Barbieri (225) in 1881. During the following 20 years Schulze and coworkers isolated phenylalanine from numerous plant species by this procedure. In 1901, Fischer (108) obtained phenylalanine from an acid hydrolysate of casein by fractionally distilling the ethyl esters of the amino acids and fractionally crystallizing the amino acids liberated by baryta hydrolysis from the ether soluble portion of the highest boiling ester fraction. Although the phenylalanine was partly or completely racemized essentially this method was applied to numerous proteins by other workers during the succeeding 30 years. In 1940, Baptist and Robson (18) isolated 0.3% of phenylalanine as the pierolonate

from a casein hydrolysate after precipitating a fraction containing leucine and tyrosine. Phenylalanine picrolonate was also obtained by these investigators in 1.7% yield from the monoamino acid fraction of casein hydrolysate prepared by the butanol extraction procedure of Dakin.

15. Proline

Proline is isolated in 15% yield from gelatin by the method of Bergmann (27). Arginine is removed from gelatin hydrolysate as the flavianate, proline is precipitated as the rhodanilate, the crude product is recrystallized from HCl-containing methanol and decomposed with aqueous pyridine and the crude proline is recrystallized from ethanol and ether. Bergmann and Niemann isolated proline from fibrin (30) and hemoglobin (31) essentially by this procedure. Neuberger (200) isolated proline as the picrate from the mother liquors obtained in the isolation of hydroxyproline from gelatin but no experimental details were given. Proline is readily isolated by Bergmann's method according to the writers' experience.

Fischer (108) isolated proline from casein in 1901. An ester fraction (b.p. 80–85°, 8–15 mm.) was boiled for 6–7 hours in five volumes of water, the amino acids were fractionally crystallized from water and water-ethanol, the filtrate containing most of the proline was evaporated to dryness and the crude mixture of L- and DL-proline was converted to the copper salts and fractionally crystallized from absolute ethanol. The copper salt of L-proline was found to be more soluble in absolute ethanol than that of the DL-form. Essentially this method has been employed by other workers for the isolation of proline from numerous proteins. In most instances, the proline isolated was impure or the degree of purity was not established.

Proline is isolated (as the picrate) in about 10% yield from gliadin by the method of Town (256). The sulfuric acid hydrolysate of gliadin is freed from sulfate ions, the amino acids in the filtrate are precipitated as copper salts, the copper salts are granulated with acetone and extracted with dry methanol, methanol is removed from the extract, the residue is dissolved in water and copper is removed from the solution as CuS, the filtrate is evaporated and the suspended tyrosine is removed, an alcoholic solution of cadmium chloride is added, and the precipitate is dissolved in water, cadmium and chloride ions are removed and picric acid is added to the filtrate. The precipitated proline picrate is recrystallized from water. According to Cox and King (67), the proline may be recovered in 90%

yield by suspending the picrate in aqueous-aniline solution, extracting the proline solution with ether, decolorizing the aqueous layer, evaporating the filtrate, dissolving the residue in absolute ethanol and adding ether to the alcoholic solution. Dakin (71) isolated about 9.5% of proline by butanol extraction of the sulfuric acid hydrolysate of gelatin. The more insoluble amino acids (leucine, phenylalanine and hydroxyproline) were removed, the butanol was evaporated, the residue was extracted with ethanol, the ethanol was evaporated, the aqueous solution of the residue was treated with mercuric acetate and barium hydroxide to remove traces of other amino acids and the filtrate was evaporated. By the procedure of Kapfhammer and Eck (158), arginine is removed from a gelatin hydrolysate as the flavianate and proline is precipitated first as the reineckate and then as the cadmium chloride complex. The yield is 3.7%.

16. Serine

Serine is isolated in 7.5% yield by the method of Stein, *et al* (236a, 237, 238). The HCl hydrolysate of technically degummed silk is freed from chloride ions, tyrosine is precipitated and removed, and glycine is precipitated as the 5-nitronaphthalene-1-sulfonate. Methyl cellosolve is added to the filtrate and alanine is precipitated as the azobenzene-*p*-sulfonate.* Barium acetate is added to the filtrate, the precipitated barium salts are removed, barium ions are removed from the filtrate and yellow serine *p*-hydroxyazobenzene-*p'*-sulfonate is precipitated and recrystallized from water. The serine sulfonate is decomposed with barium acetate, the barium sulfonate suspension is filtered, barium ions are removed from the filtrate and serine obtained from the final filtrate is recrystallized from water and alcohol.

Serine was isolated from an ester fraction (b.p. 100–125°/0.5 mm.) of an HCl hydrolysate of degummed silk by Fischer and Skita (119) in 1902. Although the product was DL-serine, essentially this procedure has been employed by other workers for the isolation of serine from numerous proteins. Serine has been isolated from proteins as the β -naphthalenesulfonate by Abderhalden and Voiti-novici (9), Schuwirth (229) and other workers. The isolation of serine through its oxazoline and benzoyl derivatives has been described recently by Elliott (100a).

* See Stein and Moore (236c) for preparation of *p*-hydroxyazobenzene-*p'*-sulfonic acid.

17. Threonine

Threonine was isolated in 0.08% yield from fibrin by the method of McCoy, *et al.* (198). Commercial fibrin was hydrolyzed with sulfuric acid, the sulfate ions were removed, the filtrate was evaporated, the suspension of precipitated amino acids was filtered, basic cupric carbonate was added to the filtrate and the precipitated copper salts were removed. Copper was removed from the filtrate as CuS, the filtrate was evaporated and extracted repeatedly (17 times) with large volumes of butanol, and butanol was removed from the extract. The residual amino acids were dissolved in water, sulfuric acid and phosphotungstic acid were added. The precipitate was removed, and the filtrate was evaporated, the precipitate was removed, the sulfuric acid and the phosphotungstic acid were removed from the filtrate, ethanol was added, and the crude threonine precipitate was recrystallized from water and ethanol.

The isolation of threonine through its oxazoline and benzoyl derivatives has been described recently by Elliott (100a). The yield of L-threonine from 5 g. of casein was 40–45 mg.; representing about 1% of the protein and about 20% of the L-threonine present in the hydrolysate.

18. Thyroxine

Thyroxine was first isolated from thyroid glands by Kendall (160, 161). Although improvements were made later (162), the yield of thyroxine was only about 0.5 g. per pound (0.11%) of thyroid. Kendall's thyroxine, as well as the products isolated by Harington (140) and Foster (124), were optically inactive owing to racemization which occurred during alkaline hydrolysis of the thyroid tissue. L-Thyroxine was isolated from enzymatic hydrolysates of thyroid by Harington and Salter (143).

Physiologically active thyroxine is isolated from the alkaline hydrolysate of iodinated proteins by the method of Ludwig and von Mutzenbecher (182). Casein (or other protein material) is iodinated, essentially according to the method of Hofmeister (150), by the action of iodine at 40° in the presence of sodium bicarbonate. The product obtained by precipitation with HCl is centrifuged, washed, dissolved in NaOH and dialyzed until free iodine has been removed. The reprecipitated material, containing about 7.4% of iodine and 125 guinea pig units of thyroxine per

gram, is refluxed with barium hydroxide and thyroxine is isolated from the hydrolysate as the sodium salt. Thyroxine is prepared by dissolving the sodium salt in alkaline 80% ethanol and acidifying the solution with acetic acid. Thyroxine has been prepared by other workers (12, 142, 212) essentially by this procedure. According to Reineke and Turner (213), optically inactive thyroxine is formed, equivalent approximately to 30% of the tyrosine in the protein, by incubating at 60° a mixture of the protein and two atoms of iodine per mole of tyrosine present in the protein in mildly alkaline solution in the presence of a compound of manganese. The report of von Mutzenbecher (197) that thyroxine is formed by incubating 3,5-diiodotyrosine in alkaline solution has been confirmed by other investigators (213). It has been reported that a synthetic thyroprotein (Protomone) stimulates growth, metabolism and the production of milk, milk fat and eggs (262).

19. Tryptophan

Tryptophan is isolated from casein in 0.7% yield by Cox and King's (68) adaptation of the procedures given by Hopkins and Cole (151), Dakin (70) and Onslow (203). Commercial pancreatin is added to an aqueous suspension of commercial casein, sodium carbonate and sodium fluoride. The mixture is saturated with toluene and allowed to stand at 37° for five days. It is shaken daily during this period. Additional pancreatin is added and the mixture is allowed to stand for 12 days. The precipitate which forms on standing overnight in the refrigerator is reserved for the preparation of tyrosine. Sulfuric acid is added to the filtrate, the solution is cooled, a solution of HgSO_4 in sulfuric acid is added, the mixture is allowed to stand for 48 hours and the supernatant liquid is siphoned. The yellow residual material is washed thoroughly first with a sulfuric acid solution of HgSO_4 to remove tyrosine and then with distilled water to remove sulfuric acid. The moist precipitate is suspended in water, a hot solution of Ba(OH)_2 is added until the mixture is alkaline to phenolphthalein and the mercury ions are removed as HgS . Barium ions are removed as BaSO_4 , the filtrate is evaporated *in vacuo* and the aqueous solution is extracted repeatedly with *n*-butanol. The butanol solution of tryptophan is distilled *in vacuo*, the residual material is cooled and filtered, the crude tryptophan is dissolved in hot 60% ethanol, insoluble material is removed, the ethanol solution is decolorized with norit and the purified tryptophan is washed with ethanol and ether. Trypto-

phan has been isolated from various proteins by this method even though the isolation and purification procedures are long and tedious.

Sahyun (221) has recently described a method for the large-scale preparation of crude tryptophan by selective adsorption on carbon.

20. Tyrosine

Tyrosine is isolated from casein in 3% yield by the method of Cox and King (68). The precipitate reserved for the preparation of tyrosine (tryptophan procedure, page 241) is suspended in 2.5 N HCl, the suspension is boiled for 30 minutes and strained, the filtrate is heated with norit, the hot suspension is filtered and the warm filtrate is extracted with benzene to remove fat which retards filtration. The aqueous solution is heated to boiling, the solution is neutralized with ammonia, the mixture is allowed to stand overnight in the refrigerator and the suspension is filtered. The crude tyrosine is dissolved in hot NaOH solution, the solution is decolorized with norit, the hot suspension is filtered, and the warm filtrate is neutralized with HCl. The mixture is allowed to stand overnight in the refrigerator, the suspension is filtered and the tyrosine precipitate is washed free of chloride ions.

Cox, *et al.* (69) isolated 22 grams of tyrosine from 4 kilograms of blood corpuscle paste (containing 5.80% nitrogen and 36.2% solids). The paste was hydrolyzed with sulfuric acid, sulfate ions were removed as BaSO₄, the filtrate and washings were concentrated *in vacuo*, the residual liquid was allowed to stand for two days in the refrigerator, and the suspension was filtered. The crude precipitate was boiled with glacial acetic acid to dissolve leucine, the purified tyrosine was dissolved in NaOH, the solution was acidified with HCl and the tyrosine precipitate was washed free of chloride ions.

The isolation of tyrosine from HCl and H₂SO₄ hydrolysates of casein and other proteins was first reported by Bopp (50) in 1849. Since that date tyrosine has been isolated from corn gluten (130, 186), casein (18, 20), and silk and silk proteins (fibroin and sericin). Silk is the best source of tyrosine since it may contain as much as 13% of this amino acid. Yields varying from about 5 to 11% of tyrosine have been reported by workers who hydrolyzed silk or silk fibroin with sulfuric acid (112, 118, 157), hydrochloric acid (1, 2, 8), formic acid (285) and papain (33). Silk waste is hydrolyzed with hydrochloric acid, the dark-colored suspension is filtered and washed with boiling water, excess HCl is distilled *in vacuo* from the

combined filtrate and washings, the syrup is diluted with water and decolorized with norit or Nuchar XXX, the suspension is filtered and the filtrate is neutralized to about pH 6 with 3 *N* NaOH. The crude tyrosine is purified by dissolving in 3 *N* NaOH and adding 3 *N* HCl to pH 6. The precipitated tyrosine is washed with boiling water and is finally purified by dissolving it in boiling water and cooling the solution. The yield is usually about 6%.

21. Valine

Valine was first isolated from lupine sprouts by Schulze and Barbieri (226) in 1883. Asparagine, proteins and leucine were removed and valine was separated from phenylalanine by fractional crystallization of their copper salts. This procedure was used by Schulze (224) in the isolation of valine from various types of plant sprouts. Fischer and Dorpinghaus (114) first removed glutamic acid (hydrochloride), tyrosine, leucine, the diamino acids (phosphotungstates) and glycine (ethyl ester hydrochloride). The remaining amino acids were esterified, the esters were fractionally distilled, and valine was separated from leucine, phenylalanine and other amino acids by fractional crystallization of the copper, lead and zinc salts. Modifications of these methods have been employed by other workers who isolated valine from various proteins.

V. PURIFICATION

Analytically pure amino acids may be prepared by recrystallization from water and from aqueous solutions of methanol, ethanol and other solvents. A summary of conditions employed in the writers' laboratory for the purification of the common naturally-occurring amino acids is given in Table VII. Selection is made of the solvents, temperatures and other conditions which will effect maximum purification with minimum loss of material. Purification is continued until the amino acids are analytically pure as established by the criteria outlined in Section VI.

It has been found advantageous in preparing high purity amino acids to use only C.P. acids and bases, redistilled organic solvents, decolorizing carbon which has been boiled with 6 *N* HCl to remove acid-soluble impurities and washed with water to remove HCl, and sintered-glass, Hirsch-type or Buchner-type funnels equipped with special (low ash, high wet strength, acid washed, lintless) filter paper (such as Eaton-Dikeman's Lintless filter papers, no. 852-4). Flasks should be scrupulously cleaned and kept covered so far as

possible. Solutions and suspensions should be mixed by shaking or rotating the containers rather than by stirring the mixtures. During crystallization flasks should be shaken at intervals to prevent caking and to facilitate formation of uniform-size crystals. Amino acid precipitates should be washed until tests are negative for chloride ion or other known soluble impurities. Precipitates should be blown

TABLE VII
SUMMARY OF CONDITIONS FOR PURIFICATION OF
NATURAL AMINO ACIDS

Amino Acid	Solvent 1		Solvent 2		Wash Fluid ^e
		ml./g. Amino Acid		ml./g. Amino Acid	
Alanine	water	3.0	95% ethanol	6.0	95% ethanol
Arginine	water ^a	1.2			40-95% ethanol
Arginine · HCl	0.4 <i>N</i> HCl ^b	0.5			80-95% ethanol
Aspartic acid	water	25.0			ice water
Cystine	1.5 <i>N</i> HCl	18.0	0.6 <i>N</i> NH ₄ OH to pH 5		hot water
Glutamic acid	water	12.0			50-100% ethanol
Glycine	water	2.3	95% ethanol	1.0	35-95% ethanol
Histidine	water ^a	6.0			50-95% ethanol
Histidine · HCl · H ₂ O	0.2 <i>N</i> HCl	1.0			75-95% ethanol
Hydroxyproline	50% methanol 50% ethanol	3.1			methanol
Leucine ^c	water	24.0	95% ethanol	24.0	50-95% ethanol
Lysine · HCl	0.2 <i>N</i> HCl ^b	1.0			60-95% ethanol
Methionine	water	17.0	95% ethanol	34.0	80% ethanol
Proline	abs. ethanol ^d	15.0			abs. ethanol
Tryptophan	65% ethanol	42.0			95% ethanol
Tyrosine	water	200			ice water

^a Carbon-dioxide free.

^b HCl is used to depress hydrolysis of the amino acid hydrochloride. Lysine · HCl · 2H₂O crystallizes from the HCl solution but the water of crystallization is lost at 45°.

^c Product employed should have been purified by one of the methods described previously (page 236). Purification by means of the formyl derivative is tedious and the yield is low.

^d A mixture containing 75% *n*-propanol and 25% ethanol (95%) may be used.

^e The first washing is made with the solvent of lowest concentration and the last with that of highest concentration.

from containers and kept separate from any residual material. Purified amino acids should be preserved in scrupulously clean dry bottles which, so far as possible, are kept tightly stoppered with glass stoppers or covered with plastic caps.

VI. CRITERIA OF PURITY

An amino acid sample thought to be pure should be a white solid devoid of any suggestion of color and it should yield a crystal-clear solution free entirely from specks of carbon, shreds of filter paper or toweling, or other visible insoluble impurities. It has been found futile to analyze further any sample which does not conform to this standard. Subjection of the sample to the qualitative tests described by Stoddard and Dunn (240) and Dunn and Rockland (85) is an informative second step in the analysis. If the proportions of ammonia and the inorganic ions are below the allowable limits

it can be reasonably expected that the high purity of the product will be borne out by subsequent analyses. The presence of an excessive quantity of any one of these impurities indicates that the product should be further purified.

The degree of purity of a purified natural amino acid is determined by establishing within acceptable limits a) the moisture content, b) the theoretical total nitrogen, amino nitrogen or carboxyl content, c) the specific rotation and d) the solubility. In special cases, determination of purity by microbiological assay or by chromatographic analysis is desirable, if not obligatory. Descriptions of certain of these analytical procedures are given below.

Methods have been described for the determination of amino acids by the Van Slyke gasometric nitrous acid and ninhydrin procedures (85) and by photometric ninhydrin techniques (191a).

1. Moisture

A glazed porcelain crucible is heated for 5 hours at 70° and 10 mm. in a vacuum oven in the presence of fresh anhydrous calcium chloride. The crucible is placed in a desiccator over anhydrous calcium chloride and, after 30 minutes' cooling, it is weighed at three one-minute intervals. The weights are plotted and the curve is extrapolated to zero time. The heating and weighing procedures are repeated until the extrapolated weight is constant within 0.5 mg. An accurately weighed 1 to 1.5 g. of sample of the amino acid is transferred from the weighing bottle to the crucible. The crucible is heated for 24 hours, cooled and weighed under the described conditions. Moisture may be determined satisfactorily in most amino acids but glutamic acid should be heated below 50° to avoid dehydration to pyroglutamic acid. Histidine monohydrochloride monohydrate does not lose its water of crystallization when it is heated at 75° *in vacuo*. Only air-dried samples of amino acids should be employed for moisture determination since oven-dried products are often too hygroscopic to be maintained moisture-free and to be weighed accurately.

2. Ash

A glazed porcelain crucible of approximately 25-ml. capacity is heated for 3 hours in a muffle furnace at 600°. After the crucible has cooled to about 90° it is placed for 60 minutes in a desiccator over anhydrous calcium chloride. The crucible is weighed using the technique described in part 1. The heating and weighing proce-

dures are repeated until the extrapolated weight is constant within 0.2 mg. An accurately weighed 1 to 1.5 g. sample of the amino acid is transferred from a weighing bottle to the crucible. The crucible is heated in the muffle furnace for three hours at 600° after which it is cooled and weighed in the manner described. Ash has been determined satisfactorily by this method in nearly all of the amino acids. This method is essentially the same as that described by Chibnall, *et al.* (59).

3. Total Nitrogen

Most of the amino acids may be determined satisfactorily by the Kjeldahl method of Miller and Houghton (189). The amino acid sample (0.5 to 1.5 mg.) is digested six hours after clearing with a mixture containing 500 mg. of potassium sulfate, 50 mg. of mercuric oxide and 1.5 ml. of concentrated sulfuric acid. Comparable results may be obtained using an amino acid sample containing 3–10 mg. of nitrogen and the apparatus, the digestion mixture (2 ml. of a sulfuric acid solution containing 5 mg. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 0.5 mg. of powdered selenium) and the experimental procedure described by Ma and Zuazaga (185). A review of the Kjeldahl nitrogen method has been given recently by Kirk (164a).

4. Titration

Aspartic acid and glutamic acid may be titrated accurately with standard base using a glass electrode to determine the end point. Although the other amino acids are too weak to be titrated directly by this method, the acid strength is increased about four-fold by adding formaldehyde to aqueous solutions of the amino acids. Under these conditions, these amino acids may be titrated with high accuracy using Dunn and Loshakoff's (84) modification of the classical formol-titration procedure of Sørensen (234). A highly sensitive glass-electrode apparatus (218) is required and technical difficulties in the preparation and use of the thin-glass bulbs have restricted the determination of amino acids by this method. Dole (76) has discussed the principles and applications of this procedure.

Other titration methods which have been proposed for the determination of amino acids are potentiometric titration with a) perchloric acid in glacial acetic acid (252a) and b) base in ethylamine diamine (193a).

5. Specific Rotation

The specific rotation is a reliable index of purity of the amino acids for which standard values have been established. Reliable

values have been reported for some amino acids, but, in other cases, the specific rotations are inaccurate because of low precision of the observed rotation, impurities in the samples analyzed, and for other reasons. If the values are to be dependable within a limiting error of 0.1 to 0.2%, the purity and concentration of the solvent and the solute, the temperature, the precision of the observed rotation, and other critical variables must be adequately controlled. A summary of selected specific rotations of the amino acids considered to be most accurate and of the recommended experimental conditions is given in Table II, Chap. I.

6. Solubility

The purity of an amino acid may be determined by solubility measurements in those cases where accurate solubility data are available. The differential (Phase Rule) solubility principles employed in the determination of solubility have been outlined by Dunn and Rockland (85) (see Chap. I). The literature data considered to be most reliable for the solubilities of the amino acids in water at various temperatures are listed in Table III, Chap. I.

7. Microbiological Assay

Traces of amino acid impurities in a sample of an amino acid may be determined conveniently and with relatively high accuracy by microbiological assay methods which have been developed during the past several years. This topic has been reviewed by Snell (233) and Dunn (79a). The microorganisms most commonly used for this purpose are *Lactobacillus arabinosus* 17-5, *Lactobacillus casei* ϵ , *Lactobacillus fermenti* 36, *Streptococcus faecalis*, and *Leuconostoc mesenteroides* P-60. References to the literature on this topic have been given by Dunn and Rockland (85).

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Chapter V

CLASSIFICATION, PURIFICATION AND ISOLATION OF PROTEINS

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PROTEINS are composed of about twenty building blocks or amino acids, attached one to another primarily through the α -amino and the carboxyl group. The character of the protein is determined by the number, the kind, and the arrangement of the amino acids. Since a protein may consist of any number of amino acids residues, varying from approximately 100 up to several thousand, it is obvious that the number of possible proteins is unlimited. They occur in plant and animal tissues as complex mixtures of several proteins, together with other constituents of the tissue in various stages of association or conjugation. Until the last two or three decades, the purification of individual proteins was considered to be difficult of accomplishment. This was due largely to inadequate information regarding protein behavior and the physical chemical characteristics, labilities, and reactivities of protein molecules. Further, no methods were available to ascertain the degree of purity of protein preparations, and very few of the older preparations could be said to consist of individual proteins. With the development of solubility, electrophoretic, and sedimentation methods for determining the number of components of a protein system, fractionation of such systems and purification of individual proteins may, at the present time, be carried out systematically with assurance of success.

I. SOLUBILITY AND CLASSIFICATION OF PROTEINS

The fact that proteinaceous materials, found in plant and animal tissues, consist of mixtures of proteins which vary in solubility characteristics, was appreciated from the very beginnings of protein chemistry. The separation of blood proteins into water- and salt-soluble proteins, namely into globulins and albumins, was described a hundred years ago (215). Separations were made later depending on differences in solubility in concentrated salt solutions

(195, 256, 47), in dilute salt solutions (215, 95, 171, 193), water-alcohol mixtures (172, 96, 73, 268), and in acids, and alkalies. As more and more work accumulated, dealing with seed proteins, hemoglobins, milk and egg proteins, and others, a body of knowledge was built up which led to a classification, in the early part of the 20th century, into groups, depending on their best known properties, namely solubility and composition (57). The substances consisting only of amino acids, were called *simple proteins*, while those combined with substances of other classes, were called *conjugated proteins*. The simple proteins were grouped into classes determined largely by solubility behavior, while the conjugated proteins were classified according to the class of material with which the protein was conjugated.

A. Simple Proteins

1. Albumins

Albumins are heat coagulable proteins which are soluble in water, dilute salt solutions, and dilute acids and bases. They are also characterized by being more soluble in concentrated salt solutions than other proteins, concentrations of 2 molar (0.5 saturation) ammonium sulfate or above being necessary for their precipitation. Albumins are found widely distributed in plant and animal tissues, uncombined with other substances but also in combination with small amounts of carbohydrates as in ovalbumin of egg white and serum albumin (118, 223, 183).

2. Globulins

Globulins are insoluble in pure water at their isoelectric point, but are brought into solution by low concentrations of salts and by dilute acids and alkalies (95, 171, 193, 191). In general, they are precipitated from salt solutions more readily than are albumins (below 2.0 molar ammonium sulfate). Globulins are sometimes divided into two groups, namely *euglobulins* which are the globulins as defined above, and *pseudoglobulins*. The latter group is characterized by being precipitated at a concentration of 2.0 molar ammonium sulfate but not being insoluble at the isoelectric point. The pseudoglobulins, therefore, may be termed a transition class between albumins and globulins and could as well be classified as albumins which are somewhat less soluble in concentrated salt solutions. Conversely, some typical euglobulins are not precipitated at half saturation with ammonium sulfate. Lactoglobulin is pre-

precipitated from concentrated salt solutions with the albumins, but is a typical euglobulin in all other respects.

3. Glutelins

These proteins are insoluble, at their isoelectric point, in all neutral solvents but dissolve readily in dilute mineral acids or bases. They are differentiated from globulins by the fact that they are not dissolved by salt at their isoelectric point. Typical examples are, glutenin from wheat, oryzenin from rice, and hordenin from barley (142). These are not chemical individuals but are groups of proteins which have not been fractionated into their component parts and the individual proteins characterized. Glutelins appear to be rather labile, and are prone to undergo changes during the usual procedures of extraction and purification.

4. Prolamines

Prolamines are the alcohol soluble proteins (191, 105). Their presence in plant tissue was first observed by Taddei in 1819 (246) when he separated wheat gluten into alcohol soluble and insoluble fractions. The term prolamines was suggested for the alcohol soluble proteins by Osborne (190), since on hydrolysis they yield large amounts of proline and ammonia, the latter indicating a high content of amide nitrogen. The prolamines are insoluble in water and absolute alcohol, but soluble in 70 to 80% aqueous ethyl alcohol. Examples are, zein from corn, gliadin from wheat and rye, hordein from barley, and the alcohol soluble proteins found in rice, kafir, and sorghum. All of the prolamines contain lower amounts of lysine than most proteins. The solubility of prolamines in 70 to 80% ethyl alcohol, is a reflection of the fact that, compared to other proteins, they contain many non-polar side chains and relatively few polar groups in the molecule. Prolamines show a low buffering capacity toward alkalies or acids, also indicating a scarcity of polar groups (105).

5. Albuminoids or scleroproteins

The scleroproteins are animal proteins which form the principal organic constituents of the skeletal structures and the protective covering of the animal body, e.g., elastin from ligament, collagen from tendon, hide, and bone, and keratin from horn, hoofs, and feathers. In general, they are characterized by their marked insolubility in all neutral solvents, and by the fact that they are dispersed

with difficulty in any solvent without decomposition. Collagens are resistant to peptic and tryptic digestion but are readily converted to gelatin by boiling with water. Elastins are digested by trypsin but not by pepsin, while keratins resist digestion by trypsin or pepsin and are insoluble in all solvents. On hydrolysis with acid, many of the keratins yield large amounts of cystine. Keratin has been defined by Block and Vickery as, "a protein which is resistant to digestion by trypsin and pepsin, which is insoluble in dilute acids and alkalis, in water, and organic solvents, and which, on acid hydrolysis yields such quantities of histidine, lysine, and arginine that the molecular ratios of these amino acids are respectively 1:4:12" (28).

6. Histones

Histones are basic proteins which are soluble in water but insoluble in dilute ammonia solution. In molecular size, they stand intermediate between the protamines and the higher proteins. They are found in animal tissues combined with acidic substances such as heme and nucleic acid (125). On hydrolysis, they yield a predominance of basic amino acids and especially lysine and arginine (125). With other proteins, histones form insoluble complexes. They are coagulated on heating but the coagulum dissolves readily in dilute acids. Examples are, globin from hemoglobin (125), thymus histone (154, 14, 109), and scombrone from mackerel sperm (125).

7. Protamines

Protamines are similar to histones in being basic water soluble proteins which precipitate other proteins and in yielding on hydrolysis, predominantly the basic amino acids arginine, lysine, and histidine (125, 177). They are, however, of comparatively low molecular weight and might be classified as polypeptides (125, 198, 259, 155). They are not coagulated by heat. All of the known protamines have been isolated from the sperm of fishes, i.e., salmine from salmon sperm, sturine from sturgeon sperm, clupeine from herring sperm, and scombrine from mackerel sperm. They are present in the sperm in combination with nucleic acids forming nucleoproteins (125, 177, 237, 179). Kossel (125) has separated the protamines into three classes, depending on the basic amino acids present: class 1 contains only the basic amino acid arginine; class 2

contains two basic amino acids, arginine and either lysine or histidine; and class 3 contains all three of the basic amino acids. Arginine is present in all protamines. When these proteins are mixed with nucleic acids, in neutral solution, insoluble nucleoproteins are precipitated.

8. Solubility as a basis of classification

Classification of a group of substances of the number, variability, and complexity, of the protein molecules, based on solubility, is inadequate and cannot describe with exactness all proteins. The weaknesses of this system become more obvious as knowledge of protein behavior increases. Gradations in solubility from one class to another occur, and difficulties arise in placing all proteins in the type classes. Probably, the most obvious example of this difficulty comes to light in consideration of albumins and globulins. Globulins are differentiated from albumins by the fact that those classified as "true" or euglobulins are insoluble in water at the isoelectric point whereas albumins are soluble. The question arises however, how insoluble or how soluble? Globulins themselves vary in solubility at the isoelectric point, so the distinction becomes one of degree only. Further the so-called pseudoglobulins have the solubility properties of the albumins in water, but their solubilities in concentrated salt solutions lie in the region empirically assigned to the globulins. Low concentration of salt effects the solution of the globulins insoluble at the isoelectric point. However, the interaction of salt at low concentration to increase the solubility of proteins, is common to both albumins and globulins, and is not a distinguishing characteristic except as related to the base level of solubility at the isoelectric point, in the absence of salt. In a solvent other than water, such as aqueous alcohol in which albumins are only slightly soluble, the presence of salt increases the solubility markedly, and the albumin in this solvent behaves like a globulin in water (75).

In spite of the recognized weaknesses of the present system, the classification of the simple proteins based on solubility is perhaps as good a criterion as any in reflecting their over-all physical chemical properties. While the conception of protein molecules as dipolar ions (263) and definition of the isoelectric point of proteins (176) followed, in point of time, classification of the proteins, it is clear that differentiation, based on solubility, can hold only at the iso-

electric point, i.e., when the proteins are in comparable physico-chemical states. At other hydrogen ion concentrations, the solubility properties are those of protein salts, and these are different from the solubility of the uncombined protein. The isoelectric point, therefore, enters into the classification insofar as it determines the point of solubility comparison. The isoelectric point, in terms of pH value, is a definite physical constant for each protein, but it cannot be used, as such, as a basis of differentiation of protein classes, since proteins of obviously different properties may have similar isoelectric points, and conversely, proteins of widely different isoelectric points may have other properties which are similar.

Amino acid composition of proteins, the relationship of polar and non-polar groups, and the magnitude of the dipole moments of protein molecules, also determine the solubility of proteins, and hence are factors in the protein classification as originally based on solubility behavior. Solubility of amino acids in alcohol, and in water, is related to the relative proportion of non-polar groups in the molecule. Thus, proline with many methylene (CH_2) groups is the most alcohol soluble of any of the amino acids. Presumably the same is true for proteins, since the alcohol soluble prolamines yield a high proportion of proline on hydrolysis and are therefore rich in parafine side chains. Of those proteins which are rich in polar groups, those which are soluble in water at the isoelectric point (albumins which have been investigated) have been found to have small dipole moments, while those that are insoluble at the isoelectric point have large dipole moments with presumably high crystal lattice energies which prevent dispersion in solvents except by interaction with salt (53).

B. Conjugated Proteins

The conjugated proteins, as the name implies, are combinations of proteins with substances which are not proteinaceous. They yield on hydrolysis, other substances than amino acids, such as carbohydrates, lipids, and a number of other moieties. These groups are usually bound to the protein in such a manner that they may be separated from the protein without disrupting the protein molecule. The exact nature of the linkage is, however, largely unknown. Many of the conjugated proteins are known to perform specific functions in the animal organism, e.g., hemoglobin, hemocyanins, "yellow enzyme," and the nucleoproteins which are found widely distributed in every cell.

1. Phosphoproteins

Phosphoproteins are perhaps the simplest of the conjugated proteins. They contain phosphorus, combined through ester linkage with the hydroxyl groups of the hydroxy amino acids such as serine or threonine. In the intact phosphoprotein, such as casein, the phosphorus is not released through the action of animal alkaline phosphatase.¹ On tryptic hydrolysis of the phosphoproteins, various phosphorus containing degradation products, such as peptides or peptones, are obtained. On treatment of the degradation products with phosphatase, the phosphoric acid is set free. Proteins may be phosphorylated by treatment with phosphorus oxychloride (165a, 203, 204, 205, 156, 157).

2. Mucoids (Glycoproteins)²

These are the carbohydrate containing conjugated proteins. They are, in general, amorphous substances of high molecular weight. The carbohydrate moieties are complex mucopolysaccharides which always contain acetyl glucosamine in combination with one or more of the following: galactose, mannose, rhamnose, glucuronic acid, gluconic acid, uronic acids, and sulfuric, or phosphoric acids (148, 175). The mucoids are perhaps the least well characterized of the conjugated proteins due to difficulties in extraction, isolation, and characterization. The proof of purity of most of these substances is very difficult. Certain of the mucoids, such as ovomucoid from egg white (151), seromucoid from blood serum (206), and the gonadotropic hormone obtained from human pregnancy urine (90), are very soluble in aqueous solutions. Others, such as the ovomucin B of egg white (270, 174), and mucoids of the vitreous humor and capsule of the lens (175), are almost completely insoluble and have been studied very little. The soluble mucoids are not readily precipitated by protein precipitants such as trichloroacetic, picric, or flavianic acids, nor are they as readily denatured by heat or organic solvents as are most proteins.

¹ A specific enzyme named phosphoprotein phosphatase which hydrolyzes phosphate from phosphoproteins has recently been discovered by Harris (97a).

² Meyer (175) suggests that the terms mucoids and glycoproteins should not be synonymous. He suggests that glycoproteins be reserved for the proteins containing small amounts of carbohydrate (less than 4% glycamine). This class consists mainly of certain albumins and globulins which have been shown to contain small amounts of carbohydrate. Thus, crystalline egg albumin has been shown to contain approximately 2 to 3% of a carbohydrate complex containing hexosamine, mannose, and an unidentified nitrogenous constituent (223, 183). The same is true of at least some serum albumins (118). Serum globulin preparations also contain small amounts of carbohydrates which vary somewhat, depending on the method of preparation (253, 254, 255).

3. Chromoproteins

The chromoproteins are made up in large part of the respiratory pigments found in the blood of various animals, and which perform the function of carrying oxygen to and removing the carbon dioxide from the tissues. The oxygen carrying capacity is associated with the pigmented radical, and is due to the presence of metals which are capable of being alternately oxidized and reduced.

The hemoglobins, contained in the red blood cells of vertebrates and invertebrates, are conjugates of globin, a histone, with hemin. The hemin contains 8–9% iron and is further built up of a series of pyrrole rings (209). The linkage between the protein and hemin is probably an ionic bond since it has been possible to prepare combinations of other bases, such as pyridine and nicotine, with hemin to form hemopyridine and hemonicotine respectively. The hemoglobins are readily purified and many of them crystallize very readily. All of the hemoglobins, apparently, have the same hemin (199), but the globins of the various species vary somewhat in amino acid composition (210, 27).

In the blood of some invertebrates such as crabs, lobsters, snails, squids, and octopi, the hemoglobin of the mammal is replaced by chromoproteins called hemocyanins (209, 202, 62). Copper is present in these compounds in place of iron. The hemocyanins show the typical characteristics of globulins in being soluble in dilute salt solution. The molecular weights of hemocyanins are very large. The nature of the prosthetic grouping is not clarified. It is believed by some investigators that hemocyanins contain no prosthetic group other than copper, while others hold that copper is contained in some grouping in a manner similar to the iron of the hemoglobins (62). The hemocyanins, similar to the hemoglobins, are readily purified and crystallizable, a large number having been prepared in the crystalline form (202, 99, 128, 201, 48).

Erythrocruorins and chlorocruorins are iron containing chromoproteins contained in the blood of other invertebrates, while a manganese containing pigment has been reported in the blood of the worm, *Pinna-squamosa* (89). Since chlorophyll is largely insoluble in ether until the tissue has been treated with alcohol, it is believed that it is also combined with protein in plant tissue and that the combination is broken by alcohol.

Copper has been shown to be a component of several enzymes, among them *laccase* from the lacquer tree (117), *tyrosinase* and *or polyphenol oxidase* from mushroom and potato (129, 128, 116, 61,

182), and ascorbic acid oxidase (159, 169). A copper containing protein, hemocuprein, of unknown physiological significance has been isolated in crystalline form from the red blood corpuscles of ox, sheep, and horse (164), and also from horse serum (55) and a protein which may be identical with hemocyanin has been isolated from ox liver (164). Another copper protein, also without known physiological function, is present in cow's milk (69).

4. Lipoproteins

Conjugates of lipids and proteins are found widely distributed in nature (39, 161, 162, 163). The proof of combination is, in many cases, based on non-extractability of the lipid with the usual lipid solvents, or on histological evidence. The lipids, histologically, appear to be masked and cannot be demonstrated in cells, until after treatment with enzymes or alcohol which breaks the lipid protein association (196, 30, 103, 121, 122, 29, 104).

Probably the best known example of a naturally occurring lipoprotein is the lipovitellin occurring in egg yolk (6, 37, 38, 108, 192). This protein holds approximately 18% of phospholipids in such a manner that they cannot be removed with ether. Extraction with aqueous alcohol readily removes the lipid, leaving the phosphoprotein, vitellin, lipid free. The lipid moiety consists largely of lecithin and cephalin. Another lipoprotein, lipovitellenin, which contains 36–38% phospholipid combined with phosphoprotein has recently been isolated from egg yolk (76). Alcohol also breaks up this complex.

Another naturally occurring lipoprotein, which has been studied fairly extensively, is the thromboplastic protein from beef lung tissue (40, 42, 43, 49, 50, 51, 178). Eighteen percent of lipid is bound to the protein in such a manner that it cannot be removed with acetone, ether, or chloroform, but is extractable with alcohol-ether mixtures. The lipid moiety is composed of alcohol-soluble and alcohol-insoluble phospholipids and sphingomyelin. The most active preparations obtained showed a high degree of homogeneity with respect to particle size.

The question of the type of linkage between the lipid and protein has been discussed by Chargaff (39). While it is not possible to draw any definite conclusion, the available information indicates that van der Waals' forces are predominant in holding together the components of most naturally occurring lipoproteins. Many of the lipids which are present lack the centers of attachment necessary

for covalent or electrostatic bonds. The combination often is broken by agents such as alcohol which would not be expected to disrupt electrostatic bonds. Salt formation may, however, play a part.

Synthetic lipoproteins have been prepared by reacting phospholipids with basic proteins (36, 41). The products were almost certainly due to salt formation since the basic protein salmine reacted with cephalin and phosphatidyl serine, while no reaction took place with lecithin, except at very basic reaction. Comparable differences were found when histone from calf thymus and globin from cattle hemoglobin were used as the protein reactants—2.78 and 1.03 *M*-eq. cephalin per gram of histone were bound at pH 3.0 and 7.2 respectively.

5. Nucleoproteins

Nucleoproteins are combinations of proteins with nucleic acids. The protein moieties are often the basic proteins but such is not always the case. One type of nucleoprotein, isolated from ripe fish sperm, contains protamines combined with nucleic acid (177, 179, 237). A second type are the nucleohistones which have been isolated from avian erythrocytes (125), thymus tissue (14, 109, 154) and from the sperm of various animals (125). Some of the animal and plant viruses are nucleoproteins of a third type in which the protein component is of much higher molecular weight and more complex character than the protamines or the histones. Many of the viruses have been prepared in relatively pure and some in crystalline form (Table I), and have been studied rather extensively. The amount of nucleic acid in the various viruses ranges from 5 to 40% of the total nucleoprotein (19, 146, 147, 221, 232, 233, 236). Still another series of complexes in which protein, nucleic acids, and lipids, are associated has been prepared from liver tissue and from a variety of normal and pathological animal tissues. These substances have not been as well characterized as the first three, but the components of the preparations seem to be conjugated. The lipid is not extractable with ether but can be removed with ethyl alcohol. It appears that the lipid is bound to the other components of the conjugates in a manner similar to the lipid of lipoproteins (44, 46, 88, 247, 248).

Although Miescher (177) first isolated nucleic acid (and protamine) from fish sperm, the nucleic acids which have been most extensively studied are those prepared from thymus gland and from yeast, the former representing the animal and the latter plant

nucleic acids (114, 149). Yeast nucleic acid contains the purines, adenine and guanine, and the pyrimidines, cytosine and uracil; the bases in thymus nucleic acid are the same except that thymine replaces uracil. When nucleic acid is subjected to mild hydrolysis by dilute alkali, four different types of *nucleotides* are obtained, each identical except for the base, which is different in each case. The nucleotides are composed, in addition to the base, of one mole each of sugar and phosphoric acid (87, 149, 150).

The manner of linkage between the protein and the nucleic acid appears to be salt-like between the basic groups of the proteins and the acid group of nucleic acids. This linkage may be, and undoubtedly is, modified by the nature of the substances in question, through non-polar linkages. Thus in the protamine and histone type of nucleoprotein, the union is readily broken by the addition of neutral salt such as sodium chloride. It would appear that only polar linkages are involved in this case. When the protein component is more complex, neutral salt does not readily achieve separation of the two components until after treatment with substances such as urea or guanidine hydrochloride which have a denaturing effect on the protein-nucleic acid complex (22, 23, 87).

C. Denaturation and Degradation Products

Further classification has dealt with altered proteins and with the products of digestion of proteins. The terms *proteans*, *metaproteins*, and *coagulated proteins* represent attempts to differentiate between the denatured insoluble products resulting from the action of water, dilute alkalies and acids, and from heat, alcohol, etc., respectively. The products of digestion of proteins by acids, alkalies or enzymes are termed *proteoses*, *peptones*, and *peptides*. Proteoses are precipitable by saturated ammonium sulfate whereas peptones are not, while peptides are defined as any combination of two or more amino acids.

II. PURIFICATION AND ISOLATION OF PROTEINS

A. General Considerations

As knowledge has accumulated regarding protein behavior, it has become apparent that proteins, in general, are labile substances which may readily undergo incompletely understood changes even under mild conditions of temperature, pH, etc. These are referred to as denaturation changes (see Chapter IX). Experience has indicated the general conditions under which these alterations are

least likely to occur for the various types of proteins. Successful purification, isolation, and crystallization of native proteins require painstaking work with strict attention to detail in carrying out the specialized techniques, under working conditions which have been found by experience to be most conducive to protein stability.

1. Temperature

The rate of denaturation of proteins increases with rising temperature. In many cases, denaturation goes on fairly rapidly even at room temperatures, and it is desirable when attempting to extract and purify an unknown protein to carry out all procedures at as low a temperature as feasible. A temperature just above the freezing point of water is usually used. Not only is a low temperature desirable from the standpoint of protein stability as such, but enzyme action, resulting in degradation, and bacterial growth with consequent putrefactive changes, are much reduced.

The precaution of working at low temperatures is of the utmost importance when organic solvents such as alcohol or acetone are to be used as solvents or as precipitating agents. Such solvents have much the same denaturing effect on many proteins as heat, producing coagulation and insoluble products. This effect is very much reduced at low temperature, and if the temperature is sufficiently low they may be prevented entirely. Egg albumin, which is readily denatured by alcohol at room temperatures, has been shown to be readily crystallizable after being dissolved in 25% alcohol at -5° for a month (75).

2. Hydrogen ion concentration

The pH of the solution has a very marked effect on the stability of proteins and must be carefully controlled. The pH of maximum stability is, however, an individual characteristic which must be determined for each protein. Some proteins are stable over a wide range of pH, others are susceptible to acids or to alkalies, while still others are stable only over very narrow pH ranges. In general, the region of greatest stability for most proteins is, as would be expected, near neutrality, which is the pH of the natural environment of most proteins. Conditions of mild acidity and alkalinity should therefore be maintained in dealing with solutions of proteins whose properties are unknown.

3. Effect of salt on protein stability

Protein stability is increased by the presence of inorganic salts, particularly so when the solutions are relatively dilute with respect

to protein. This fact is of practical value, since most proteins are soluble in dilute salt solutions, and may readily be extracted from tissues with such solvents. Extracted in this manner, the proteins are more likely to retain their native properties than in salt-free solvents. After concentration, proteins which are soluble in the presence of moderate concentration of salt may be stored for relatively long periods of time without change. Also, if wet protein precipitates are to be stored, their stability is increased by covering with a moderately concentrated salt solution. The effect of salt on proteins in solution may be due to several factors, chief of which is the specific interaction of the inorganic ions with the charged groups of the protein dipole ion.³

4. Effect of protein concentration on stability

Proteins are more stable in concentrated solutions than when the protein concentration is low. Concentrated solutions (20 to 40%) of albumins may be kept almost indefinitely under sterile conditions, with no detectable change in their properties. Denaturation changes are favored in dilute solutions resulting in the formation of either insoluble coagulated products or of lower molecular weight fragments due to dissociation of the protein. Increased stability with increasing concentration is probably due to increased dielectric constants and to interaction of the protein dipoles with each other. The effect is similar to that of the presence of salt.³

5. Surfaces and interfaces

Many proteins are readily denatured at surfaces, in films, or at interfaces between two liquids. A common illustration of this fact is the result of beating egg white. Protein solutions foam readily and denaturation takes place under those conditions. It is desirable, when protein solutions must be stirred, or when they are being poured from one container to another, that the operation be carried out without the creation of foam.

6. Drying of proteins

Proteins are more stable when dry than when in solution. It is therefore desirable, whenever possible, to store proteins in dry form. The process of drying is best carried out from the frozen state (lyophilization). The solution is frozen on the inner walls of a flask which is then attached to a condenser immersed in alcohol-

³ For a discussion of the theory of the interaction of proteins with other ions, the reader is referred to Cohn and Edsall (53) and Scatchard and Kirkwood (214).

solid CO₂ mixture or similar freezing mixture and the system evacuated. The moisture is sublimed, while the loss of heat is rapid enough to keep the protein solution frozen during the process. Very little if any denaturation occurs for most proteins, as contrasted with other methods of drying. Protein powders of large surface area are obtained which are easily redissolved and are thus convenient products with which to work.

Protein solutions may be partially concentrated before lyophilization and after dialysis by suspending the cellophane tubes containing the protein solution in the cold room and circulating the air past them with a fan. Evaporation through the membrane is fairly rapid, especially if the diameter of the tubes is small.

Protein in solution, after being made salt free by dialysis, may be precipitated and dried by cold acetone or alcohol. The cold protein solution is poured slowly, with stirring, into four or five volumes of acetone or alcohol which has previously been chilled to -5° . The precipitate is collected by centrifugation or filtration in the cold, washed two or three times with dry cold (-5°) acetone or alcohol, and the residual organic solvent immediately removed *in vacuo*. This method involves the risk of denaturation of some proteins, and should not be used except when lyophilization facilities are not available.

7. Collection of precipitates

Collection of precipitates may be accomplished either by centrifugation or by filtration. Filtration is satisfactory for crystalline or granular protein precipitates but amorphous protein preparations filter too slowly to make this a desirable procedure. In general, filtration on Buchner funnels with suction is the method to be preferred, since it is possible to remove the mother liquors far more completely than by centrifugation or by gravity filtration. The funnels should be of such a size that the final filter cake is relatively thin (5 mm. or less). A suitable hardened filter paper should be used to facilitate the removal of the filter cake. As the filtration nears completion, care should be taken to prevent cracks from forming, permitting the passage of air, foaming, and local drying of the precipitate. This may be done by pressing the moist filter cake with a spatula where cracks begin to form. A more satisfactory method is to cover the funnel, after all the solution is on the filter, with rubber dam firmly fastened around the edges of the funnel. Air is thereby prevented from passing through the precipi-

tate, and the pressure of the rubber dam prevents cracks from forming. In no case must filtration be allowed to proceed until the cake becomes dry, since air drying is detrimental to essentially all proteins.

Centrifugation is often used for collection of precipitates and it has certain obvious advantages in handling and manipulation of the sample, particularly if the precipitate is small or if the volume to be handled is very large and filtration is difficult. Centrifugation usually necessitates more thorough washing of the precipitates, with solvent of the same pH and salt concentration, to remove the mother liquors contained in the precipitate. The final precipitate, after washing, still contains far more solvent and consequently more salt (if present) than if the material was filtered. It is often convenient, if the volume is large, to first collect the precipitate by centrifugation, but remove the final wash liquors by filtration to obtain the precipitate in a more compact and solvent free form.

Separation of an aqueous protein extract of a tissue from the insoluble residue is often a difficult and tedious procedure, depending on the nature of the tissue being extracted. The best method to be used has to be determined for each individual case. Filtration, either by gravity, using fluted filters, or by suction in Buchner funnels is often satisfactory as is also centrifugation, either in a batch type or in a Sharples centrifuge. Straining through cheesecloth or other coarse filters may be desirable to remove the major part of the insoluble residue. Addition of filter aids to the solution and filtration through filter aid or paper pulp filters is also often useful, but the possibility of adsorption of the protein must be considered. A persistently "muddy" or opalescent solution may be flocculated by a slight pH adjustment, or the addition of salt up to 0.8 to 1.0 *M*. ammonium sulfate may cause precipitation or coagulation of the offending material, together with small amounts of protein, leaving a clear solution for further fractionation. If it is desirable to recover the protein from the precipitate, this can usually be done since the volume has been reduced and more rigorous filtrations, etc., may be used if necessary, to effect the removal of the offending material.

8. Dialysis

Dialysis as a means of separating crystalloids and colloids was first introduced by Graham (79). The procedure is widely used in protein chemistry for various purposes, but most generally to remove salts and other electrolytes from protein solutions. The

protein solution is introduced into bags or closed tubes of collodion or cellophane, through the walls of which the electrolytes will diffuse while the proteins, due to their large molecular size are retained. The membranes, containing the protein solutions, are suspended in water and the dialysis allowed to proceed until near equilibrium has been established. It is apparent that, to remove essentially all the salt from the solution, a very large volume, or many changes of water, will be necessary. This inconvenience can be obviated, and the time required to remove the salt very much reduced, by conducting the dialysis against distilled water which is constantly being renewed, thereby maintaining the maximum concentration gradient across the membrane. When the protein solution is believed to be salt free the flow of water is stopped, and after a few additional hours of dialysis, the water washing the membranes is tested for the ions which are being removed. If a positive test is obtained, the dialysis is continued until no salt can be detected.

The constant renewal of the water may be simply accomplished by using as the dialysis vessel, a tall glass jar which is equipped with an inlet tube at the bottom and an overflow tube near the top. By combining a number of such jars in series, the water may be used to bathe membranes containing protein solutions of increasing salt concentration. A stream of air, bubbling through the water, effectively stirs both the protein solution inside the membranes and the outside solvent. Other more elaborate methods, such as circulation of water through a series of glass tubes containing the membranes, and also counter flow of the protein solution and the wash water, have been devised and used. The speed of the dialysis is greater the smaller the diameter of the membrane tubes, due to the greater surface in proportion to volume of solution.

The membranes which are almost universally used at the present time are made from cellophane tubing which may be obtained in a variety of sizes. The tubing is first cut to the desired length and one end closed by a triple fold of $\frac{1}{2}$ to one inch, depending on the size of the tubing, followed by longitudinal folding and securing the folds by winding with tightly stretched rubber bands of the proper size. After introduction of the protein solution, the second end is closed in a similar manner. For the smaller sized tubing, the ends may be closed by simply tying a knot. This has the disadvantage of inconvenience when opening the membrane after the dialysis is completed, since no excess unfilled tubing is available for handling as is the case when a folded end is opened.

Before introducing the protein solution, the membranes must be tested for leaks. This is most simply done by filling with water and exerting pressure on the filled membrane. Defects will be apparent by a fine stream of water. A more rigorous test is to fill the membrane with a non-diffusible dye and suspend in water. No dye will appear on the outside of the membrane if it is perfect.

Dialysis to remove salt should be carried out at as low a temperature as possible to assure stability of the protein, especially when the solution becomes essentially salt free and particularly when the protein concentration is also relatively low. In addition to low temperature, special precautions should be taken to prevent bacterial growth. This may be done by saturating the water with toluene, or other effective preservative, by passing the water through a toluene wash tower before it enters the dialyzing vessel.

Dialysis is useful for purposes other than the removal of electrolytes from the protein solutions. The procedure may be used to advantage, whenever it is desirable to add reagents slowly and in such a manner as to avoid local excess in the solution. Increasing the salt or alcohol concentration in salting out and precipitating procedures, changing the pH of the solution, and adjusting protein solutions to definite low ionic strengths by dialysis against buffers of the appropriate pH and ionic strength, as in globulin fractionation, may all be carried out effectively. Dialysis is useful in the crystallization of proteins to effect a gradual change in conditions of pH or other composition of the solvent, in order to produce a slight but gradually increasing degree of supersaturation. Some of these special uses will be discussed in later sections.

The method of pressure dialysis originally used by Sørensen (225) is useful, not only as a method of removing electrolytes but also simultaneously concentrating the protein solution. The fact that the solution becomes progressively more concentrated is of advantage in increasing protein stability as already discussed. Specially constructed collodion membranes or cellophane membranes supported by an outer jacket of some strong porous material such as fine linen must, however, be used to withstand the pressure.

Electrodialysis is used to obtain a protein solution as free of electrolytes as possible. The procedure consists of placing the protein solution in a compartment separated from the electrodes by membranes and passing a current through the solution. The water around the electrodes is constantly renewed to avoid concentration of the products of electrolysis. The dialysis is continued until the conductivity of the protein solution falls to a constant level. Practi-

cally, electrodialysis is not used except after removal of essentially all electrolytes by ordinary dialysis. Unless this is done and if the current is not carefully controlled, the heating effect may be excessive, resulting in denaturation. Many designs of equipment have been used, some of which are commercially available (107, 120).

B. General Purification Procedures

Proteins vary in their physical chemical properties such as their acidic or basic behavior, isoelectric points, dielectric properties, the magnitude of the dipole moments, the crystal lattice energies, and in chemical composition. These differences are reflected in their solubility in water, in acids or bases, in organic solvents such as alcohol, and in salt solutions, either concentrated or dilute. These differences are the basis of methods by which proteins may be separated from one another and isolated in pure form. Due to the peculiarities of each individual protein, however, no one procedure or set of conditions will suffice in its entirety for the extraction, purification, and isolation of more than one protein. Each protein, or system of proteins, presents its own problem, which must be solved by study of the particular protein or set of proteins. However, the variations are in most cases ones of degree rather than of kind, and the application of one or more general procedures, with necessary modifications, will usually bring about the desired fractionation. Careful and accurate observation of the behavior of the protein in the solution or extract under question will usually point out the changes in the conditions which are most likely to yield the desired result.

1. Preparation of sample for extraction

Proteins occur in plant or animal tissues together with other constituents from which they must be extracted and separated. Occasionally as in blood serum or plasma the proteins are already in a media from which they may be precipitated and concentrated directly, but this is the exception rather than the rule. For most plant and animal tissues, the proteins must be obtained in solution before fractionation or purification can be accomplished. An efficient extraction can only be carried out on finely ground tissue. For most tissues, the disintegration can be carried out merely by grinding the tissue in the cold as rapidly as possible, and extracting the ground tissue immediately in order to prevent microbial or enzymic deteriorative changes.

Freezing tissue in solid carbon dioxide and grinding the tissue

while still frozen is a procedure which is to be recommended for the preparation of many tissues for protein extraction. This process is sometimes difficult to carry out, however, where large quantities of some types of tissues are to be processed. Muscle tissue must be dissected into small pieces or strips before freezing, in order to grind after freezing. Many plant tissues may readily be handled in this manner. Before beginning grinding operations a certain amount of solid carbon dioxide must be passed through the grinder in order to chill the equipment and prevent thawing of the frozen tissue. Solid CO_2 is also mixed and ground simultaneously with the tissue in order to maintain the low temperature. The CO_2 is allowed to evaporate after the grinding is completed and the cold tissue is then ready for extraction.

Tissues may be prepared for subsequent protein extraction by drying with acetone. The ground tissue is extracted two or three times with three to four volumes of cold acetone and finally washed with cold ether and dried. The finely divided residue can then readily be extracted with protein solvents. This procedure is sometimes desirable when the tissue contains large amounts of fatty material which, if allowed to remain, seriously interferes with aqueous extractions. If the extraction with the organic solvents is carried out in the cold (0 to -5°), only the most sensitive proteins will undergo denaturative changes. Pituitary tissue, for example, may be prepared in this manner with very little if any destruction of the protein hormones. This procedure may also have the advantage of arresting enzyme action, as was found to be the case in the extraction of insulin. It was found necessary to immerse the pancreatic tissue in alcohol, immediately after removal, in order to prevent enzymic destruction of the hormone (15, 16).

2. Extraction of proteins

All proteins, with the exception of the scleroproteins, are soluble in either water, dilute salt, aqueous alcohol, or acid or basic aqueous solutions. Different fractions may be obtained by extracting the tissue with the various solvents and this has often been done in the past. In general, however, it is advantageous to obtain as many as possible of the proteins in solution with the same extracting medium, and then separate the various proteins by appropriate methods. Extracts are thereby obtained which are more concentrated than if the older procedure is used, thereby decreasing the possibility of change. Further, it is possible to use dilute salt solutions in which protein stability and solubility are usually greater

than in water or in dilute acids or bases. Dilute (1-3%) solutions of sodium chloride, sodium sulfate, or ammonium sulfate, adjusted by the addition of the corresponding acid or alkali, to the optimum pH value for extraction, are most generally used. If proteins of more specialized solubility characteristics such as alcohol soluble proteins, lipoproteins, or proteins soluble only at extreme conditions of acidity or alkalinity, are present, these may be obtained in solution with aqueous alcohol (50 to 90%), more concentrated salt solutions (5 to 10%), and strongly acidic or basic solutions, generally after removing the more commonly occurring proteins as indicated above. If, as is often true, the aim of the experimentation is the extraction of a biologically active protein, the procedure employed will be governed by the solubility and stability of the protein as indicated by retention of activity. Good protein technique will, however, yield the best results in those cases concerned with isolation and crystallization.

Putrefactive changes are prone to occur during the course of extraction with aqueous solvents, even though the operation is carried out in the cold. To minimize this possibility, the extracting medium may be saturated with some preservative such as toluene, previous to extraction. Excess toluene in the system, resulting in emulsion should, however, be avoided.

The isolation of an individual protein, and particularly the isolation of a physiologically active protein, requires the extraction of large amounts of source tissue. The hormone, enzyme, etc., is invariably present in small amounts, which, during the course of purification, becomes concentrated to progressively smaller amounts by weight. If sufficiently large amounts of original material were not extracted, it would become impossible to concentrate the protein sufficiently and to manipulate the small amount of concentrate with the degree of accuracy necessary, as regards the control of pH, salt concentration, and other variables.

3. Separation of proteins by "Salting Out" procedures

Fractional precipitation of proteins by neutral salts has long been a conventional procedure (31, 32, 45, 55, 77, 78, 82, 83, 106, 111, 180, 228, 229, 230). Salting out has been stated to be due to the stronger attraction of the salt for the water, removing it from the protein and hence forcing the protein out of solution (66, 67, 106). The effectiveness of the salts depends on the valency of the anions, sulfates and phosphates being more effective precipitants than

chlorides, acetates or nitrates. Saturation with sodium chloride precipitates globulins from solution but not albumins. Half saturation with ammonium sulfate or potassium phosphate precipitates the majority of the globulins while full saturation results in the precipitation of the most soluble albumins. The solubility of the salt also must be considered. While sodium sulfate is as effective a precipitant as ammonium sulfate at equivalent concentrations, its solubility is low except at elevated temperature, and many proteins cannot be precipitated except by increasing the temperature above that usually employed or which is recommended. Ammonium sulfate, on the other hand, precipitates the most soluble proteins even near the freezing point of water. While not so commonly used, potassium phosphate is an excellent protein precipitant. It is soluble to almost the same extent as ammonium sulfate and the buffering power of phosphates is of advantage in maintaining constant pH. Carbonates are effective salting out agents but, because of their alkalinity, are suitable only in special cases.

The solubility of proteins in concentrated salt solutions is controlled by several factors. It varies with pH and passes through a minimum in the region of the isoelectric point, the exact pH value of minimum solubility usually being somewhat different from the true isoelectric point (see Fig. 7, Chap. VII). Separation of two proteins by salt precipitation may, therefore, be better effected under certain conditions of pH than others, depending on the influence of pH on the character of the solubility curves of the two proteins in concentrated salt.

The solubility of proteins in concentrated salt solutions varies also with the temperature. The temperature coefficient of solubility may be either positive or negative depending on the protein (72, 82, 189, 228) (Fig. 1). The solubility of myosin (72) and carboxy hemoglobin (82, 86), for example, increases, that of egg albumin varies little, while the solubility in salt solutions of seed globulins (189) and lysozyme (7) decreases with decreasing temperature. Change of temperature may therefore become an effective means of precipitating one protein while retaining the other in solution. Particularly is this the case when the protein components have opposite temperature coefficients of solubility.

The relation of salt concentration and protein solubility has been formulated by Cohn (52) in the equation:

$$\log S = B - K_s \Gamma / 2$$

where S is the protein solubility, $\Gamma/2$ is the ionic strength, and B and K_s are constants. If $\log S$ is plotted against the ionic strength, B is the logarithm of a hypothetical protein solubility at zero salt concentration obtained by extrapolation of the line back to the ordinate axis. K_s is the salting out constant which determines the slope of the line, and measures the rate of solubility change with change in salt concentration. It is independent of pH and temperature and is characteristic of the protein and the salt. B on the other

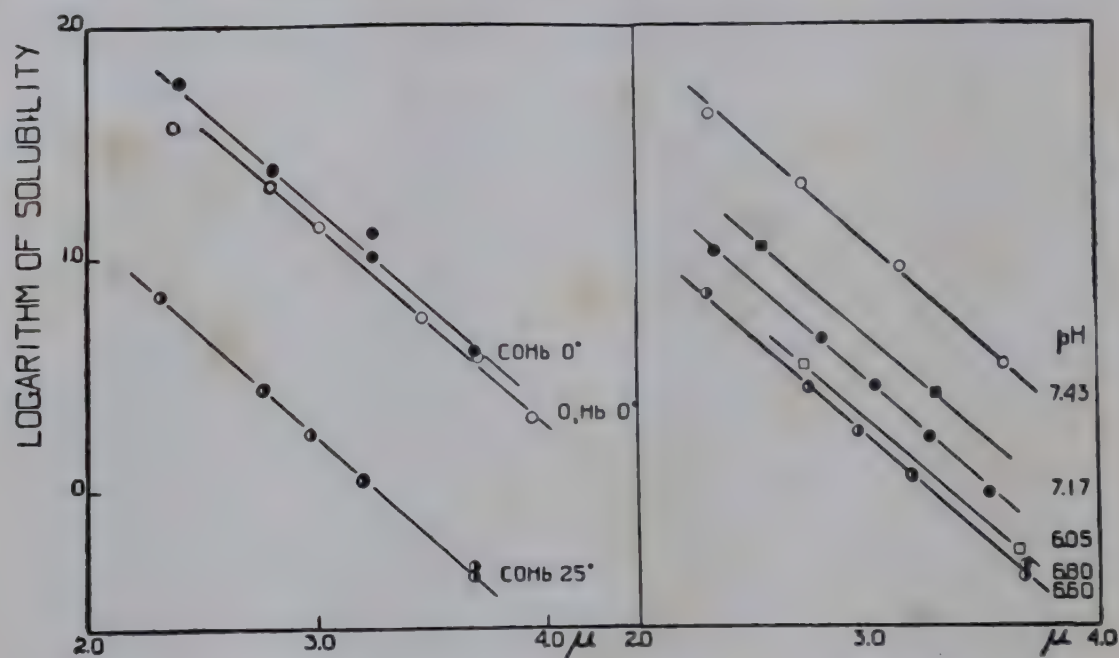


FIG. 1. The solubility of hemoglobin in concentrated phosphate buffers of varying temperature and pH. (From Green, A. A.: *J. Biol. Chem.*, 93:517, 1931.)

hand is markedly affected by both pH and temperature (Fig. 1). This linear relation holds only when the ionic strength is high and the solubility of the protein small.

Salt may be introduced into the protein solution in several ways. The solid salt may be added directly to the solution to the required concentration. Unless the salt is added slowly with adequate stirring, local concentration of salt will occur which may interfere with the separation and may result in denaturation of the most sensitive proteins. The precipitation of the protein by this method is rapid, but the rapid flocculation of the protein may lead to excessive occlusion of other proteins, and the flocculent nature of the precipitates makes centrifugation or filtration difficult.

The salt may also be added in the form of its saturated solution.

In this case, the addition should again be as slow as possible, and with stirring to avoid local excess of salt. Devices by which the salt solution is introduced beneath the surface of the solution in a fine spray have been used effectively.

A third and perhaps the best method from several standpoints of introducing salt into protein solutions is by dialysis (167). The saturated solution or a saturated solution containing an excess of solid salt is contained in a cellophane bag which is suspended from the shaft of a motor in the protein solution (Fig. 2). The bag is slowly rotated, thereby stirring the protein solution as well as the salt solution. The water and salt diffuse through the membrane in opposite directions until equilibrium is reached. The rate of increase of concentration can be adjusted, within limits, to the desired speed by controlling the porosity of the membranes and the concentration gradient across the membrane. No local concentration of salt takes place and the character of the protein precipitates is improved, in that they tend to become more granular and are therefore collected more readily. Separations tend to become more precise, and duplication of fractions is facilitated. The chief disadvantage is the time required for equilibrium to become established, but in most cases this is outweighed by the advantages mentioned.

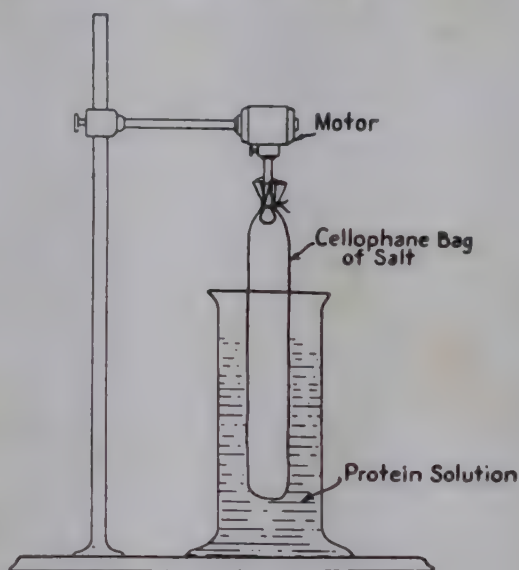


FIG. 2. Diagram of the apparatus used for adding reagents to protein solutions. (From McMeekin, T. L.: *J. Am. Chem. Soc.*, 61:2284, 1939.)

4. Separation of proteins by isoelectric precipitation and "Salting In" procedures

The proteins which are insoluble at their isoelectric point in the absence of salt are the glutelins and the true globulins. These may be separated from the more soluble proteins, such as the pseudoglobulins and the albumins, by isoelectric precipitation. Glutelins and globulins, when they occur together, are readily separated since the globulins dissolve in dilute salt solutions at their isoelectric point while the glutelins are insoluble in all neutral solvents.

The usual procedure for the purification of the globulins of a protein system is to effect a separation from the more soluble proteins by precipitation with salt (0.5 Sat. $(\text{NH}_4)_2\text{SO}_4$), redissolving the precipitate, and removing the salt by dialysis. The pH is then adjusted to the point of maximum precipitation and the euglobulin precipitate removed. If more than one euglobulin is present, and if their isoelectric points are sufficiently far apart, more than one zone of precipitation may be observed and the corresponding globulins may be separated. The pseudoglobulins remain in solution, and may be purified by salt or alcohol fractionation procedures.

The separation of individual euglobulins from one another may or may not be readily accomplished. Differences in solubility of globulins are due to 1. differences in isoelectric point, 2. differences in "salting in" or "salting out" effect, 3. variation in their interaction with other proteins in solution, and 4. differences in temperature coefficient of solubility. Of these, differences in the isoelectric point, and differences in their interaction at the isoelectric point with salt at low ionic strength, have been most often used in effecting their separation.

Various globulins have markedly different solubilities in the presence of salt of low ionic concentration. The degree to which an isoelectric globulin solution must be made "salt free" in order to precipitate individual globulins, varies with the individual character of the globulin, some precipitating when the salt concentration has been reduced to a low level, either by mild dialysis or by dilution, others require exhaustive dialysis, while to effect the precipitation of some globulins, the solution must be made as salt free as possible by electrodialysis. Conversely globulins may be separated from one another by extraction of isoelectric precipitates with buffers of increasing ionic strength. The solvent action of neutral salts varies, increasing roughly in proportion to the square of the valence of the salt cation.

In addition to the effect of salt on the solubility of the globulins, the presence of proteins has a solubilizing effect due to the interaction between proteins. It is, therefore, usually beneficial to separate the globulins from the major part of the soluble albumins by salt precipitation, before proceeding to the isoelectric fractionation. Further, if two globulins are present in solution with isoelectric points fairly close together, the point of maximum precipitation may be somewhere between the two proteins and both may precipitate at a pH which is not the isoelectric point of either. If the effect

of salt on their solubility is also nearly the same, the separation of the two becomes difficult. The solution must then be made salt free at a pH, either alkaline or acid to the isoelectric point of the globulins, and the pH raised or lowered until a precipitate appears. The precipitate will consist predominantly of the protein whose isoelectric point is first approached. By repeated precipitation, an effective separation may usually be made (Fig. 3) (84).

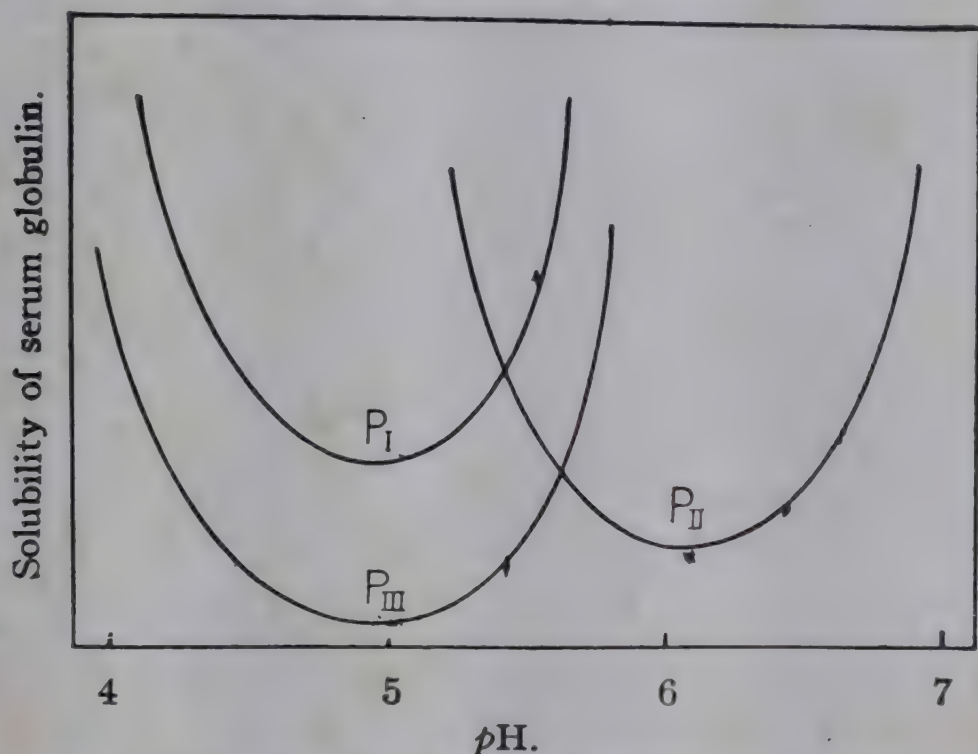


FIG. 3. Separation of serum globulins by isoelectric precipitation.
(From Green, A. A.: *J. Am. Chem. Soc.*, 60:1108, 1938.)

The specificity of the effect of salt at low ionic strength may also be utilized in separations of globulins at other pH values than the isoelectric point. The addition of acids or alkali to a point slightly on either side of the isoelectric point may result in a marked increase or decrease in solubility, depending on the nature of the protein and also on the nature of the salt (82, 94, 227).

Separation and fractionation of the water insoluble but salt soluble globulins, from one another, is dependent on the specific electrochemical properties of the globulins. Interaction with electrolytes at low concentration is a reflection of the variation in dielectric increments, crystal lattice energies and dipole moments of the proteins (123, 214). The solubilizing effect of salt increases roughly in proportion to the square of the dipole moment. These forces, therefore, vary many fold from protein to protein, and result in

wide variation in solubility. The variation in the solubility of protein in concentrated salt solution, on the other hand, is very much less, since the specific electrochemical forces are masked in high salt concentration and are not the determining factors. The theoretical possibilities of separating globulins by "salting in" are, therefore, much greater than of fractionating the water soluble proteins by "salting out" (56).

5. Separation of proteins with organic solvents

Proteins are precipitated from solution by organic solvents which are miscible with water, such as acetone, or methyl and ethyl alcohols. This fact has been used as the basis for separating various proteins by many investigators (54, 96, 158, 172, 173, 226, 269). The solubility of egg albumin, for example, is reduced from 40% in aqueous solution to 0.01% in 25% alcohol at -5° (75). Recently, ethyl alcohol has been used in the fractionation of serum proteins and in the preparation of individual human serum proteins for therapeutic use (54, 56, 186a, 243a). The order of precipitation of the serum proteins is the same as for salt precipitation: serum

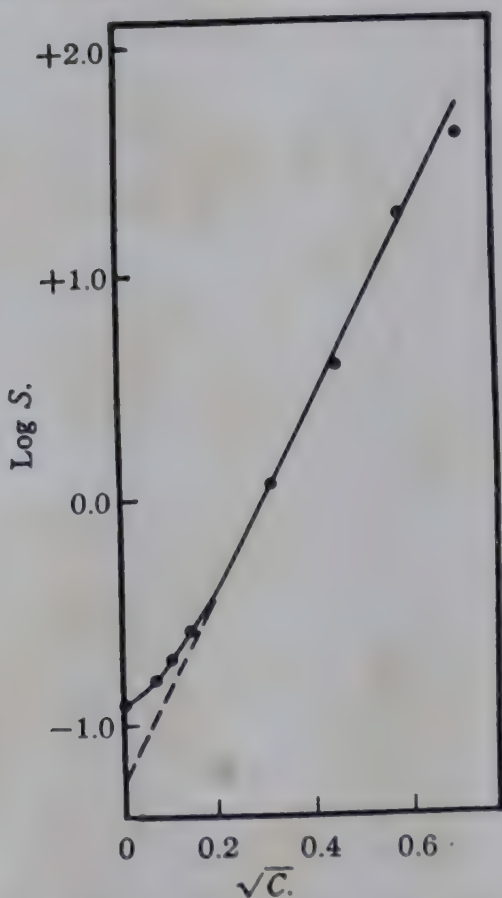


FIG. 4. The influence of NaCl on the solubility of egg albumin in 25% ethanol at -5° . (From Ferry, R. M., Cohn, E. J., and Newman, E. S.: *J. Am. Chem. Soc.*, 60:1480, 1938.)

globulins precipitate in the presence of 20 to 40% alcohol while the albumins require 40 to 50%.

The advantage of working with proteins in alcohol-water mixtures and the increased possibilities of effecting fractionations have been discussed by Cohn, *et al.* (56). Practically, the necessity of extensive dialysis to remove salt is obviated, the precipitates may readily be dried at low temperature, and sterile conditions are maintained during the procedure. The use of alcohol-water mixtures makes it possible to utilize, not only the precipitating action of the alcohol, but also the specific interaction of the water soluble proteins with salt at low ionic strength, in effecting protein separations. The presence of small amounts of salt in the system has the same solubilizing effect on the water soluble proteins in alcohol-water mixtures as has salt on euglobulins in water at their isoelectric points (54, 75) (Fig. 4). Since the degree of interaction of protein with salt at low ionic concentration is specific for each protein and depends on the electrochemical characteristics of the protein, the separations themselves become more specific and "salting in" becomes a valuable tool for separating water soluble proteins. By balancing the precipitating action of the alcohol with the solubilizing effect of the salt, a number of conditions for the separation of individual proteins may be set up. When the variable effects of pH and temperature on protein solubility in such systems are also considered, the possibilities of protein fractionation in alcohol-water mixtures are further increased.

The practical utility of the alcohol-water system for fractionation of proteins on a large scale has been effectively demonstrated by the preparation of human plasma albumin for therapeutic use in the armed forces during the recent war. It is certain that the processing of the large volume of human blood plasma collected by the Red Cross could not have been carried out on the same scale by salt fractionation procedures. The full value of aqueous alcohol and other similar systems for protein purification has only recently been appreciated and an increasing use of such procedures in investigational work and in commercial processing, seems certain.

6. Purification of proteins by adsorption methods

Adsorption procedures for separation and purification of proteins have not been as generally used as other methods which have been discussed. They have, however, been used in cases where the special adsorptive properties of the protein in question have indicated the use of adsorbents as a means of effecting separation from other

proteins or as a means of concentration (7, 115, 134, 141). When adsorption procedures are to be used in the purification of a protein, the process must be studied with respect to selection of the most suitable adsorbent (permutite, silica, aluminum hydroxide, bauxite, bentonite, ion exchange resins), the pH of adsorption, and of elution and the selection of the most efficient eluant. For the elution process, aqueous solvents containing neutral salts, inorganic or organic buffers or mixed solvents such as aqueous pyridine or other water soluble organic acids or bases may be used.]

Since, as has already been pointed out, many proteins are readily denatured at surfaces, adsorption procedures cannot be used indiscriminately. Thus while adsorption methods have been used in the purification of enzymes, particularly by German chemists, they have been found by Northrop (185) not to be generally useful in the purification of enzymes, due to marked losses of activity. Losses may also occur due to incomplete adsorption or more likely to incomplete elution with the relatively mild solvents which are suitable for protein work.

It is probable that, due to the development of synthetic ionic

TABLE I

CRYSTALLINE PROTEINS		
PLANT PROTEINS		
Name	Source	References
Avelin	Oats	187
Concanavalin A	Jackbean	239
Concanavalin B	Jackbean	239
Edestin	Hemp seed	207
Excelsin	Brazil Nuts	165
Globulin	Castor bean	188
Globulin	Flax seed	188
Globulin	Grapefruit seed	18
Globulin	Jackbean	243
Globulin	Lemon seed	18
Globulin	Orange seed	212
Globulin	Pumpkin seed	188
Globulin	Sesame seed	207
Globulin	Wheat seed	186
Globulin	Watermelon seed	127
Phycoerythrin	Algae	140
Phycocyan	Algae	140
Protein	Yeast (3)	135, 136
Protein	Latex of <i>Antiaris toxicaria</i>	126
Protein	Potato	112, 124
Purothionin	Wheat	12, 13
Virus protein	Bean	200
Virus protein	Cucumber	20
Virus protein	Tobacco	231, 234
Virus protein	Tomato	21, 235

TABLE I—*Continued*

ANIMAL PROTEINS

Name	Source	References
Apo-ferritin	Ferritin	81
Bence-Jones Protein	Urine	197
Chlorocruorin	Polychaete worms	211
Ferritin	Liver and Spleen	80
Globulin	Urine (2)	93, 110
Globulin	Liver	69a
Globulin	Pathological blood serum	257
Hemocyanins	Invertebrates	48, 99, 202, 68, 128, 201
Hemocuprein	Blood corpuscles	164
Hemocuprein	Blood plasma	55
Hemoerythrin	<i>Sipunculus</i>	78, 208
Hemoglobin	Cat	264
Hemoglobin	Chicken	264
Hemoglobin	Dog	98, 264
Hemoglobin	Guinea Pig	264
Hemoglobin	Horse	98, 264
Hemoglobin	Human	33, 71, 264
Hemoglobin	Ox	264
Hemoglobin	Pig	264
Hemoglobin	Rat	264
Hemoglobin	Sheep	264
Hepatocuprein	Liver	164
Lactalbumin	Milk	267
Lactoglobulin	Milk	194
Myoglobin	Muscle, Horse	249
Myoglobin	Muscle, Human	252a
Myoglobin	Urine, Human, Donkey, Mule	209a
Myoglobin (Synthetic)	Heme plus Globin	70
Myogen	Rabbit muscle	17
Myosin	Rabbit muscle	(244, 245)
Ovalbumin	Egg white	224
Oxymyoglobin	Horse	209a
Serum Albumin	Ass	265
Serum Albumin	Chickens	143
Serum Albumin	Guinea Pig	265
Serum Albumin	Horse	265, 167, 168, 102
Serum Albumin	Human	3, 265
Serum Albumin	Mule	265

ENZYMES

Albumin	Horse serum	170
Aldolase	Rabbit and rat muscle	248a
Amylase	Sweet potato	11
Asclepain	Milkweed	34
Carbonic anhydrase	Red blood cells	218
Carboxypeptidase	Pancreas	8
Catalase	Horse liver	242
Cholinesterase (pseudo)	Serum	9
Chymopapain	Papaya latex	113
Chymotrypsin	Pancreas	138
Chymotrypsinogen	Pancreas	138
Crotoxin	Rattlesnake venom	220
Dehydrogenase	Rabbit muscle	59
Dehydrogenase, Lactic	Heart muscle	238
Dehydrogenase, Alcohol	Yeast	181
Dehydrogenase	Yeast	261
Phosphoglyceric Acid	Rabbit muscle	59a

TABLE I—Continued

Name	Source	References
Dephosphorylase	Human muscle	130
Ficin	Ficus latex	260
Fumarase	Ox heart	141
Hemolysin	Cobra venom	63, 64, 65
Hexokinase	Yeast	24, 135
Lysozyme	Egg white	2, 5, 7
Mexicain	Latex, <i>Pileus Mexicanus</i>	35
Papain	Papaya latex	10
Pepsin	Gastric mucosa, beef	137
Pepsin	Salmon	184
Pepsinogen	Gastric mucosa, beef	101
Pepsin Inhibitor	Gastric mucosa, beef	100
Peroxidase	Horse radish	251
Phosphatase	Kidney	258
Phosphorylase A	Rabbit muscle	85
Phosphorylase B	Rabbit muscle	58
Rennin	Gastric mucosa	9, 25, 26, 91
Ribonuclease	Beef pancreas	132
Trypsin	Pancreas	139, 166
Trypsinogen	Pancreas	139
Trypsin Inhibitor	Pancreas	139
Trypsin Inhibitor	Soybean	133
Tuberculin	T. B. Bacteria	219
Tyrosinase	Mushroom	60
Urease	Jackbean	240
Yellow Enzyme	Yeast	250
Zymohexase	Rat muscle	262

HORMONES

Insulin	Pancreas	217, 1
Lactogenic	Pituitary	266
Secretin	Intestinal mucosa	92
Growth	Pituitary	267a

exchange resins of various kinds with special properties, adsorption procedures for protein fractionation may become more widely used. Theoretically, due to the amphoteric nature of the protein molecule, it should be possible to develop methods by which proteins of different isoelectric points could be selectively adsorbed on ion exchange resins. Thus far adsorption methods in protein chemistry probably have not received the attention and study which they merit.

7. Crystallization of proteins

A considerable number of proteins have been prepared in crystalline form either as liquid or true crystals (Table I). Many other proteins, however, have been prepared in relatively pure or in pure state by all known criteria but have not been induced to precipitate in crystalline form.⁴ The ability of a protein to crystal-

⁴ Crystallinity refers in this discussion to the formation of single crystals and not only to regularity of arrangement of molecules within the protein particle, as indicated by x-ray measurements. Most, if not all proteins are or can be prepared in crystalline form to varying degrees by the latter criterion.

lize is not known to be related to any particular physical chemical characteristic.

The conditions for crystallization of a protein seem to be specific for individual proteins since different proteins have been crystallized under a variety of conditions with respect to solvent, pH, and temperature conditions. Only very general conditions can be indicated under which crystallization may be expected to occur but no condition or set of conditions can be given which will assure successful crystallization of a protein which has not previously been crystallized.

In general, crystallization appears to be more readily induced the greater the degree of purity of the protein. Hemoglobin, which can be obtained in relatively pure form merely by hemolysis of isolated red blood cells, can be crystallized directly, but for most proteins the end is achieved only after rather extensive purification. Northrop (185) states that, in his experience, crystallization of enzymes never resulted unless all but traces of mucoid-like substances were removed and it has been found that the presence of small amounts of other impurities may inhibit crystallization. The requirement of high purity is, however, not universal as is shown by the fact that lysozyme can be crystallized in the presence of all the other proteins of egg white under the proper conditions (5).

Crystallization is more likely to occur from concentrated rather than dilute solutions of the protein (185, 167). The conditions must be such that the solutions are slightly supersaturated with respect to the amorphous form. Too high a degree of supersaturation results in a precipitation of amorphous material without crystallization. The right degree of supersaturation may be obtained by:

1. Decrease or increase of temperature
2. Addition of salt
3. Addition of organic solvents such as alcohol
4. pH adjustment
5. A combination of any of these procedures

As is the case in all crystallizations, seeding of the solutions greatly facilitates the procedure. Once having obtained a protein in crystalline form, crystallization of other preparations of the protein is facilitated by adding a few crystals to the solutions. Crystallization may then be induced under conditions of purity, etc., which will not otherwise allow crystallization without seeding.

C. Protein Preparations

1. Preparation of crystalline urease (240, 241)

- a. Extract 100 gm. defatted jackbean meal by stirring for 3 or 4 minutes with 500 ml. of 31.6% acetone (158 ml. acetone diluted to 500 ml. with water at 22°).
- b. Filter through a 28 cm. filter. When all the material is on the filter, place in cold room (2–3°) overnight. If the first portions which come through the filter are cloudy, return to filter paper.
- c. Centrifuge in the cold and wash crystals with 31.6% acetone.
- d. Dissolve crystals in 3.5 ml. water; centrifuge to remove insoluble material; and add 1.5 ml. acetone. Cool in refrigerator.
- e. Add a phosphate buffer solution, pH 6.1,⁵ drop by drop to the urease solution until the solution is turbid; about 25 drops are usually sufficient.
- f. Allow to stand overnight. Collect crystals as before.

2. Preparation and crystallization of catalase (242)

- a. Grind beef liver four times in a meat grinder.
- b. Extract with 35% dioxane (400 ml. per 300 gm. liver). Stir for four or five minutes.
- c. Place on fluted filters (32 cm., Schleicher & Schull, No. 595) and cover with watch glasses. Allow to filter into 500 ml. graduates overnight at room temperature.
- d. To each 100 ml. of filtrate, add 20 ml. of dioxane with stirring and set in refrigerator.
- e. After 12 or more hours, filter and refilter in the cold (2–3°) until solution is clear.
- f. Precipitate the catalase by adding 10.2 ml. dioxane to every 100 ml. of filtrate. Allow to stand in refrigerator overnight. Filter off the precipitate. Refilter if necessary. The residue on the filter must stand in refrigerator until all the liquid has drained.
- g. Dissolve precipitate, after removing from paper, in 3 to 15 ml. water, depending on yield of precipitate, per 300 grams of liver.
- h. Add a few drops of saliva to digest the glycogen. Filter the solution and extract the residue a second time.
- i. Chill the filtrate and add saturated $(\text{NH}_4)_2\text{SO}_4$, cautiously, until a haziness appears. The catalase crystallizes almost immediately. Keep materials in refrigerator and add more ammonium sulfate until a good crop of crystals is obtained.
- j. Collect crystals by centrifugation. Emulsify in a small amount of water and dissolve by adding the least possible volume of 9.6 per cent phosphate buffer of pH 7.4.

⁵ The phosphate buffer is prepared by adding 14.3 ml. of 0.1 M NaOH to 50 ml. of 0.2 M KH_2PO_4 and adding 30 ml. of acetone.

- k. Centrifuge off any insoluble material, and adjust to pH 5.3 by addition of KH_2PO_4 solution.
- l. Add $\frac{1}{4}$ to $\frac{1}{2}$ volume of saturated $(\text{NH}_4)_2\text{SO}_4$, slowly, to complete precipitation. Crystals may appear either as needles or as thin plates. Chilling of a concentrated solution of catalase at pH 7.4 yields prisms which dissolve in NaCl solution.

Catalase contains approximately 0.1% iron which appears to be a constituent of the molecule. Crystals resembling heme have been obtained from crystalline catalase. It is therefore a chromoprotein of isoelectric point 5.7. It is insoluble at the isoelectric point, but dissolves readily in concentrated sodium chloride solution.

3. Preparation and crystallization of lactoglobulin (194)⁶

- a. Adjust fresh skim milk to pH 4.6 with dilute HCl.
- b. Add $(\text{NH}_4)_2\text{SO}_4$ to $\frac{1}{2}$ saturation (2 molar).
- c. Filter, discard precipitate of casein and globulin.
- d. Add $(\text{NH}_4)_2\text{SO}_4$ to saturation (4 molar).
- e. Filter, and dissolve precipitate in water to make an 8 to 10% protein solution. (Approximately 1/30 of original volume of milk.)
- f. Adjust pH to 5.8 and dialyze for 10–12 days against frequent changes of distilled water. (If dialyzed against running distilled water this period of dialysis may be reduced by half.) Remove any precipitate by filtration.
- g. Adjust pH of filtered solution to 5.2, by careful addition of 0.02 to 0.05 *N* HCl.
- h. If crystalline lactoglobulin is available, seed the cloudy solution saturated with toluene with a few crystals and allow to stand for several days at room temperature. (Seeding hastens crystallization, but the oily material gradually becomes crystalline without seeding.)
- i. Recrystallize the material by dissolving in water containing NaCl (0.1*M*). Adjust pH to 5.8, dialyze as before, readjust to pH 5.2, and allow to crystallize.
- j. Dry the crystalline product by lyophilization. Approximately 1.5 gm of crystalline material should be obtained per liter of whey.

Lactoglobulin is a typical globulin which is insoluble at its isoelectric point and is dissolved at low salt concentration. It is not precipitated by $(\text{NH}_4)_2\text{SO}_4$ in the concentrations usually effective in the precipitation of globulins, but remains in the fraction which is usually considered as the albumin fraction.

4. Preparation of crystalline ovalbumin (119)

- a. Separate yolks and whites of six dozen fresh eggs.
- b. Homogenize whites by stirring with a mechanical stirrer. Avoid incorporation of air.

⁶ The procedure below is a modification of that given by Palmer (194).

- c. Add an equal volume of filtered saturated Na_2SO_4 (400 gm. per liter water) at 35° slowly without stirring. Stir slowly for two hours.
- d. Disperse 50 gm. of cellulose pulp in about 3 liters of water. Collect the pulp by filtering through cheesecloth and squeeze out water by hand. Disperse moist pulp in eggwhite solution and filter through cheesecloth.
- e. Disperse 25 gm. of cellulose pulp in the filtered solution. Disperse another 25 gm. in water and form filter pad supported by cheesecloth on a 24 cm. Büchner funnel. Filter egg white solution with gentle suction. Filtrate should be quite clear.
- f. Adjust clear filtrate to pH 4.7 with 0.5 N H_2SO_4 , added slowly with good stirring to avoid local excess of acid.
- g. Heat slowly, with stirring, to $31\text{--}33^\circ$ in a water bath maintained below 50° .
- h. Add warm ($31\text{--}33^\circ$) saturated Na_2SO_4 slowly, with stirring, to permanent turbidity.
- i. Cover solution and allow to cool slowly. After several hours, when the temperature has reached 30° stir gently. Observe "sheen" indicating crystallization. Continue stirring for three or four hours, or, conveniently overnight.
- j. Remove any insoluble material which may be present on top of the solution.
- k. Add 50 ml. of a diatomaceous earth filter aid (Celite, Super-Cel or Hy-flow) to each liter of ovalbumin suspension. Filter with gentle suction through a porous filter paper (Whatman No. 4) in a 24 cm. Büchner funnel until filter cake is fairly dry.
- l. Extract filter cake six successive times with 500 ml. of water filtering off extract each time and combine filtrates.
- m. Warm ovalbumin solution to $31\text{--}33^\circ$ as in g. Add warm saturated Na_2SO_4 with stirring as in h and repeat i, j, k, and l.
- n. Dialyze final solution until salt free and dry by lyophilization.

5. Preparation and crystallization of lysozyme by an adsorption procedure (7)

- a. Add 150 ml. of a 10% suspension of bentonite in 1% KCl to one liter of egg white. Stir vigorously for three to five minutes avoiding foaming.
- b. Collect the clay by centrifugation.
- c. Wash clay with 300 ml. 0.5 M phosphate buffer, pH 7.5. Centrifuge and discard supernatant.
- d. Wash clay 3 times with 300 ml. (total 900 ml.) 5% aqueous solution of pyridine. This removes inactive adsorbed proteins. Discard solutions.
- e. Elute lysozyme by washing the clay twice, each time with 300 ml. of a 5% aqueous solution of pyridine, which has been ad-



FIG. 5. Lysozyme hydrochloride crystals. (From Alderton, G. A., and Fevold, H. L.: *J. Biol. Chem.*, 164:1, 1946.)

justed to pH 5.0 (glass electrode) by the addition of sulfuric acid. For best results, elution should follow adsorption within 24 hours.

- f. Dialyze eluates against running tap water until no odor of pyridine remains. Finish dialysis against running distilled water for 24 hours.
- g. Dry the protein solution from the frozen state. The product is essentially pure amorphous lysozyme. Approximately two grams should be obtained for each liter of egg white.
- h. Dissolve the amorphous product in water to a 5% solution.
 - (1) Adjust to pH 4.5 with HCl and 5% NaCl. Allow to stand at 4°, when crystals of lysozyme chloride are formed. (Fig. 5).
 - (2) Add 5% NaHCO₃ and allow to stand at room temperature, when crystals of lysozyme carbonate are soon deposited.
 - (3) Add 5% NaCl and adjust pH to 9.5–10.0 with NaOH and place at 4°. Crystals of isoelectric lysozyme are obtained. Note that the crystal form is different in each case.

6. Direct crystallization of lysozyme (5)

- a. Adjust pH of egg white to 9.5 with 1 N NaOH.
- b. Add sodium chloride to a concentration of 5%.

- c. Add a few crystals of isoelectric lysozyme.
 - d. Allow to stand at 4° for 24–48 hours with occasional stirring. Collect crystalline material by centrifugation.
 - e. Dissolve crystalline product in dilute acetic acid and recrystallize either by adding 5% Na_2CO_3 (pH 8.0–8.5) or by adding 5% NaCl and adjusting pH to 9.5–10.
- Lysozyme is a basic globulin which is isoelectric at pH 10.5–11.0. Its basicity is due to the presence of few acidic groups in proportion to the basic groups.

7. Crystallization of horse hemoglobin (74)

- a. Three liters of horse red blood cells, obtained from citrated horse blood, are suspended in 12 liters of 1.5% NaCl solution which has previously been cooled to 2°.
- b. Centrifuge in Sharples centrifuge at a speed of about 30,000 r.p.m.
- c. Suspend the concentrated cells in 300 ml. of water.
- d. Separate the cellular debris in the Sharples centrifuge. The solution contains approximately 25 to 28% hemoglobin.
- e. Stir solution with a Luther stirrer (an open T tube fused to a solid glass rod with an additional opening two inches above crosspiece). While stirring, adjust pH to 6.6, either by addition of $\frac{2}{3}$ M phosphate buffer in an amount to give final concentration of 0.1 M phosphate, or by the addition, drop by drop, of 0.1 M HCl . This must be carried out with care to avoid denaturation of the hemoglobin; at intervals during the addition, place drop samples on microscope slides. If the crystals formed after a few minutes are rodlike, continue adding HCl until rhomboid plates appear. Then add an additional 5 to 10 ml. HCl per 500 ml. of solution. Too much acid denatures the hemoglobin as indicated by a change in color from red to brown. From 160 to 320 ml. HCl are usually sufficient for one liter of solution.
- f. After crystallization of the oxyhemoglobin has begun, stir for 1 hour and allow to stand in cold room for an additional hour. Centrifuge in cold room.
- g. Wash two or three times by suspension in an equal volume of water followed by centrifugation.
- h. Add $\frac{1}{2}$ volume of water and stir vigorously, adding 1 N KOH , drop by drop, until solution is complete. Five ml. of base per 100 ml. of crystals is usually sufficient.
- i. Centrifuge to remove any insoluble impurity.
- j. Recrystallize as before.

The success of the procedure depends on: 1. the careful addition of reagents and particularly HCl to avoid local excess resulting in decomposition; 2. rapidity of the process (hemoglobin solutions decompose spontaneously on standing; 3. low temperature (2°); and, 4. complete oxygenation.

8. Crystallization of horse serum albumins (167, 168)

- a. Dilute fresh horse serum with equal volume of water.
- b. Bring solution to 2.1 *M* $(\text{NH}_4)_2\text{SO}_4$ by introducing the calculated amount of salt in a cellophane membrane, either as a saturated solution or as a saturated solution and solid salt, and rotate the membrane in the diluted serum. The pH should be approximately 6.6.
- c. Filter on a Büchner funnel and wash with 2.1 *M* $(\text{NH}_4)_2\text{SO}_4$. The precipitate contains serum globulins.
- d. Bring albumin solution to 2.6 *M* $(\text{NH}_4)_2\text{SO}_4$ by adding salt as before. The precipitate is largely crystalline and contains the serum albumins. Yield about 30 gm. per liter of horse serum.
- e. Dissolve in water to about a 3% solution. Refractionate at 2.1 (discard precipitate) and at 2.5 *M* $(\text{NH}_4)_2\text{SO}_4$, collecting (by filtration) the material soluble at 2.1 but insoluble at 2.5 *M* $(\text{NH}_4)_2\text{SO}_4$.
- f. Dissolve 2.5 ml. precipitate in water to a syrup, and carefully add saturated $(\text{NH}_4)_2\text{SO}_4$ until near the point of precipitation. Allow to stand overnight. Remove crystals by centrifugation. Wash with 2.0 *M* $(\text{NH}_4)_2\text{SO}_4$ and filter on a Büchner funnel.
- g. Another crop of crystals may be obtained from the original supernatant by carefully adding $(\text{NH}_4)_2\text{SO}_4$ and allowing to stand as before.
- h. Recrystallize combined crystals from f and g twice as in f and g. The final product should be free of carbohydrate. Yield 3 to 6 gm. per liter of original serum.
- i. The filtrates and washings from the carbohydrate free albumin are combined (Ca 300 ml. per liter of original serum) and brought to 2.4 *M* $(\text{NH}_4)_2\text{SO}_4$ by adding salt through a rotating membrane as before.
- j. Collect precipitate by filtration on a Büchner funnel. Dissolve to a syrup and crystallize as in f and g. The product is apparently a mixture of carbohydrate free and carbohydrate containing albumin. Store.
- k. Combine filtrates from j and completely precipitate by adding $(\text{NH}_4)_2\text{SO}_4$, through a rotating membrane, to 2.5 *M*.
- l. Dissolve in water and dialyze until salt free. Remove and discard any precipitate by centrifugation.
- m. Add $(\text{NH}_4)_2\text{SO}_4$ to incipient turbidity and collect first crop of crystals. Store.
- n. Add $(\text{NH}_4)_2\text{SO}_4$ to filtrate to the point of turbidity. Allow to stand for three or four days.
- o. Collect crystals and recrystallize three or four times, either as before, or by introducing the protein solution into a cellophane bag and rotating in a 2 *M* ammonium sulfate solution. The final product is the carbohydrate containing albumin of horse serum and contains 5.5% carbohydrate. Yield 300 to 400 mg. per liter of serum. The crystalline form of this albumin

is hexagonal plates while the carbohydrate free crystals are rod-shaped.

9. Purification and crystallization of rabbit muscle phosphorylase (85)

- a. Anesthetize well-fed rabbits by intravenous injection of pentobarbital, and bleed.
- b. Remove muscles from back and legs as rapidly, and with as little stimulation as possible. Carry out all further steps in a cold room (2 to 5°).
- c. Pass muscles through a meat grinder twice, and extract twice, with an equal volume of ice-cold water. The water is stirred into the ground muscle and allowed to stand for about 10 minutes. The extract is strained off through gauze, filtered through cotton, and then through coarse filter paper.
- d. Adjust extract to pH 6.0–6.2 with dilute HCl, and dialyze in cellophane tubes (Visking Casings, diameter $1\frac{1}{8}$ inches) against running cold water (5–10°) for three hours.
- e. Adjust pH to 5.8–5.9 with 0.03 *N* HCl (glass electrode). An isoelectric precipitate forms which flocculates readily and settles rapidly on standing, if the pH has been properly adjusted. The complete removal of this precipitate by centrifugation and filtration is an essential step in the method.
- f. Neutralize the perfectly clear solution by the addition of about 1 gram of sodium β -glycerophosphate per 100 ml. of solution.
- g. Add 0.7 volume of $(\text{NH}_4)_2\text{SO}_4$, saturated at room temperature, to make the final solution 41% saturated (1.68 molar). The $(\text{NH}_4)_2\text{SO}_4$ should be neutral and the final pH 6.8.
- h. Allow to stand overnight; decant as much fluid as possible and collect the relatively small amount of precipitate by centrifugation. Continue centrifugation until precipitate is well packed. The precipitate contains 25 to 33% phosphorylase.
- i. Suspend precipitate, obtained from the muscles of one rabbit, in 10 ml. water and dialyze against cold running water for 1 to 2 hours. During this time the proteins go into solution.
- j. Continue dialysis against several changes of glycerophosphate-cysteine buffer of pH 6–8 (1 ml. of 0.3 *M* cysteine hydrochloride plus 39 ml. of 1% sodium glycerophosphate). Place cylinder, containing dialyzing bag and buffer solution, in an ice bag. Crystals should appear in a few hours, or overnight. If the phosphorylase content of the solution was high, or if dialysis against water has been continued until the proteins begin to precipitate, the first precipitate may be amorphous. Reprecipitation in the following manner always yields crystals.
- k. Centrifuge crystals from j in the cold and immediately dissolve, at 30–35°, in a solution of 0.03 *M* cystine hydrochloride and 1% sodium glycerophosphate, adjusted to pH 6.8. Remove insoluble material (consisting partly of cystine crystals) and place solution in an ice bath.

1. Phosphorylase crystals appear, usually, within 30 minutes. This alternate solution and crystallization, by means of temperature change, may be repeated a number of times. The crystals usually appear as small pointed needles in the form of rosettes if crystallized rapidly. Upon slower crystallization, from a more dilute solution, long blunt edged needles are formed (Fig. 6).

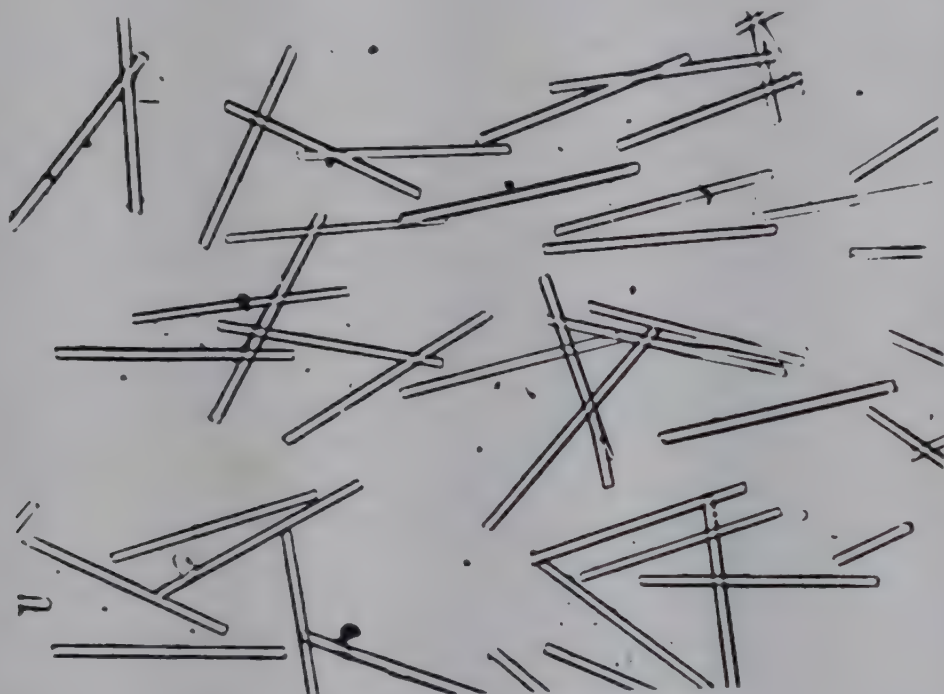


FIG. 6. Phosphorylase crystals prepared from rabbit muscle. (From Green, A. A., and Cori, G. T.: *J. Biol. Chem.*, 151:21, 1943.)

10. Purification of insulin (222)

- a. Grind fresh beef pancreas, as rapidly as possible, by passing through a meat grinder.
- b. Add 1500 ml. of 95% alcohol, and 20 or 30 ml. of 10 *N* H₂SO₄, per kilo of hash and mix well by stirring.
- c. After standing at room temperature for four to 12 hours with occasional stirring, filter on large filters. Press residue in a hand press and filter press-liquid.
- d. Evaporate the extract at low temperature (25-30°) to 1/10 of volume.
- e. Filter through moistened filter paper, and wash with small amounts of water until filtrate volume is equal to 200 ml. for each kg. of pancreas used.
- f. Add 40 gm. of (NH₄)₂SO₄ to each 100 ml. of filtrate and dissolve with stirring. On standing for some hours, the precipitate collects on the walls of the vessel and the filtrate may be poured off.
- g. Dissolve brown gummy precipitate in water to about 100 ml. for each kilo of pancreas.

- h. Precipitate by adding $\frac{2}{3}$ volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution. Collect precipitate as before.
- i. Dissolve in water with addition of 0.1 *N* NH_4OH to pH of approximately 7.0. Remove insoluble material by centrifugation, and wash with water. Dilute to 100 ml. for each kg. pancreas.
- j. Adjust pH to 5.0 with dilute acetic acid, allow to stand for three or four hours, collect the precipitate by centrifugation and wash twice with water at pH 5.0. After standing two or three days more precipitate usually forms from the mother liquors, which may be collected and combined with the main fraction.
- k. Dissolve precipitate in 0.1 *N* acetic acid (5–10 cc. per kilo of pancreas) and add 1 *N* NaOH solution to neutralize 20% of the acetic acid (pH 4.0). After standing in cold some hours, remove the precipitate by centrifugation, wash with $\frac{1}{2}$ original volume of water, and discard precipitate.
- l. To combined, often opalescent, solutions add NaOH to neutralize one half of the original acetic acid (pH 5.0).
- m. Insulin separates immediately, but for greatest recovery the solution should be allowed to stand in the cold for 2–3 days. The precipitate is collected by centrifugation and washed twice with distilled water. Dry by lyophilization.

11. Crystallization of insulin (97)

- a. Dissolve 100 mg. of the amorphous insulin powder in 25 ml. 2% acetic acid.
- b. Add 5 ml. of 6% saponin solution.
- c. Warm to 35°, add 80 ml. of 0.85% ammonia, and keep at 35° for 30 minutes.
- d. Remove precipitate by centrifugation, discard precipitate, and adjust the solution to pH 5.6 immediately, by addition of 8.5 ml. of the ammonia solution.
- e. Divide solution equally between five 15 ml. centrifuge tubes. Scratch inside of tube gently with a glass rod and allow to stand overnight.
- f. Collect precipitates by centrifugation and dissolve all five precipitates in 2.5 ml. of 10% acetic acid and 10 ml. of water. Add 2.5 ml. of the 6% saponin solution and again adjust immediately, to pH 5.6 by addition of 8.25 ml. of 0.85 ammonia. Allow to crystallize as before.
- g. Dissolve centrifuged precipitate in 4 ml. water and 0.4 ml. 0.1 *N* HCl .
- h. Pour solution into 16 ml. of phosphate buffer at pH 7.0 to which had been added 0.8 ml. of 0.1 *N* NaOH .
- i. Warm the resulting solution to 55°, add 1.0 ml. of 0.1 *N* HCl (pH 5.6) and set aside for crystallization. Yield of crystalline insulin will be from 5 to 15% of the starting material.

The first successful extraction and purification of insulin was accomplished by Banting, Best, Collip, and coworkers (15, 16). The

first crystallization of insulin was accomplished by Abel and coworkers in 1926 (1). The methods given here are modifications of the original procedures. The crystallization of insulin was noteworthy because it was the first time a protein of specific physiological action had been obtained in crystalline form. Doubt was at first expressed as to whether the crystalline product obtained by Abel and coworkers was the pure hormone or a protein containing the active molecule as an adsorbate. Another interesting fact, discovered later, is that all crystalline insulin contains a small amount of zinc (216). If all heavy metals are removed, insulin cannot be crystallized by the known procedures. Crystals have been obtained only in the presence of Zn, Ni, Co, and Cd ions. The metal appears to be a chemical constituent of the crystals and not an impurity.

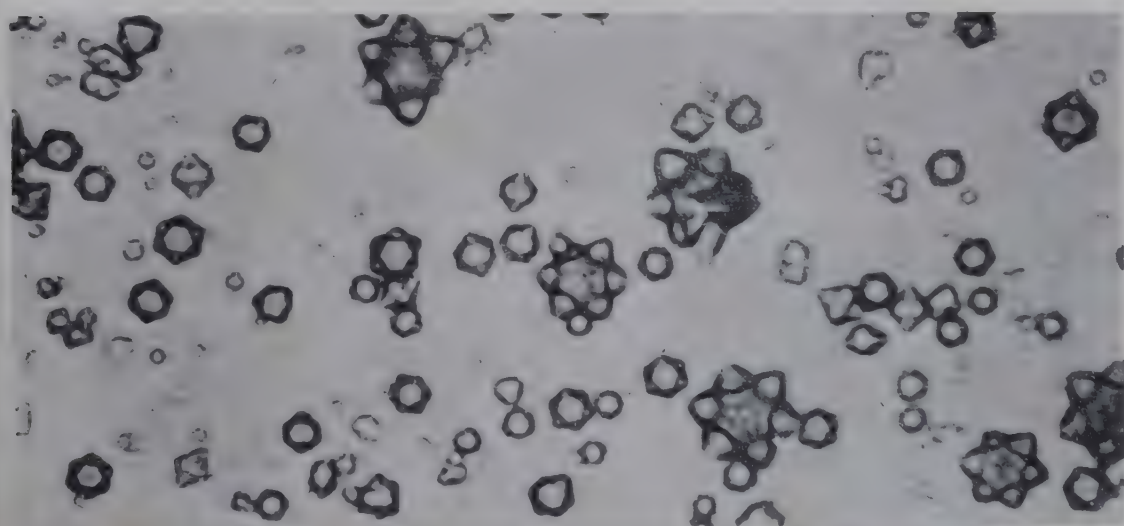


FIG. 7. Crystalline horse spleen ferritin. (From Granick, S.: *J. Biol. Chem.*, 146:451, 1942.)

12. Preparation of crystalline ferritin (80, 131, 145)

- a. Extract 1 kg. of horse spleen with 2 liters of water at 20°, for two hours. Centrifuge and extract residue a second time as before.
- b. Heat to 80°, and filter through cheesecloth and then through large fluted filters. The coagulated protein precipitate is discarded.
- c. Add 30 gm. $(\text{NH}_4)_2\text{SO}_4$ to each 100 ml. of solution. Keep at 0° overnight.
- d. Remove precipitate by centrifugation, and dissolve in distilled water (about 60–80 ml.).
- e. Add 4 to 5% of CdSO_4 and allow solution to stand overnight.
- f. Collect crystalline ferritin by centrifugation. Dissolve in 2% $(\text{NH}_4)_2\text{SO}_4$ solution to deep red-brown solution and dialyze salt free.
- g. Adjust solution to pH 4.6 with dilute acetate buffer (resulting ionic strength 0.1). Allow to stand overnight. Centrifuge and discard precipitate.

- h. Add 4–5 gm. CdSO_4 per 100 ml. solution and allow to crystallize as before. This is the cadmium salt of ferritin. (Fig. 7).
- i. The crystalline material may be freed from excess salt by solution in 2% $(\text{NH}_4)_2\text{SO}_4$ followed by dialysis until free of NH_4^+ and SO_4^{--} .

Ferritin contains more than 20% iron and, as prepared, 2–3% cadmium. Crystallization may also be effected by the addition of Zn, Ni, and Co, sulfates in place of Cd, but with more difficulty. The preparations have been found to be heterogeneous by solubility and sedimentation methods, and by variation of the iron and phosphorus content of various samples. Electrophoretically, the preparations appear homogeneous.

The phenomenon of reversible heat denaturation may be illustrated with ferritin. If a dialyzed ferritin solution is slowly heated (3 to 4° per minute), it becomes cloudy at 82°. If allowed to cool the solution clears up. If the solution is heated above 90° irreversible denaturative changes occur.

13. Preparation of crystalline apoferritin from ferritin (81)

Apoferritin is the name given to ferritin which has been freed from iron by the method given below.

- a. Place 15 ml. of a 3% ferritin solution in a cellophane bag, 1 cm. in diameter, together with 100 mg. of powdered $\alpha\alpha'$ -bipyridine.
- b. Suspend the bag in a narrow glass tube of 100 ml. capacity, containing acetate buffer at pH 4.6 and ionic strength $\mu = 0.05$.
- c. Bubble oxygen-free nitrogen through outer solution for 20 minutes.
- d. Add 100 mg. of $\text{Na}_2\text{S}_2\text{O}_4$ to outer solution. Continue bubbling with N_2 overnight.
- e. Continue dialysis against distilled water until diffusate is colorless.
- f. Add another portion of bipyridine to bag and repeat b, c, d, and e.
- g. Crystallize, by addition of 5% CdSO_4 as in 12c (Preparation of Ferritin). The crystals have a much reduced Fe content.
- h. Using the crystalline product from g, repeat the entire procedure beginning with a.

The final product should be colorless or nearly so. The crystalline form is identical with that for ferritin but the product is Fe free. Apoferritin is homogeneous by all known criteria.

14. Isolation of adrenotropic hormone (152, 160, 213)

- a. Grind 5 kg. of fresh hog pituitaries in a meat grinder. Add 5 liters of acetone containing 50 ml. of 10 N HCl to the mash gradually and put through grinder.
- b. Add 10 N HCl (about 250 ml.) to the mash to approximate pH 1.5 (thymol blue), and add 15 liters of acetone, 1 to 1.5 liters at a time with grinding after each addition.

- c. Remove insoluble residue by centrifugation or suction filtration and bring acetone concentration of extracts to 92% by addition of 30 liters or $1\frac{1}{2}$ volumes of acetone. Allow to stand overnight. Collect precipitate by decantation and centrifugation.
- d. Extract precipitate four times with 50 ml. portions of distilled water, removing insoluble material by centrifugation.
- e. Add nine volumes of acetone, and allow to stand overnight. Collect precipitate as before, wash three times with acetone, and dry in vacuo over H_2SO_4 . Yield 1 to 2 gm. per kilo of pituitary tissue.
- f. Dissolve 5 gm of precipitate in 50 ml. of water at pH 9.0, with the aid of 2 *N* NaOH.
- g. Adjust pH to 8.0 by careful addition of 0.1 *N* HCl. Remove precipitate by centrifugation and discard. Dilute supernatant with water to 235 ml.
- h. Adjust pH, successively, to pH 6.6 and 5.4 by addition of 0.1 *N* HCl, removing and discarding precipitates.
- i. To the supernatant at pH 5.4, add 17 ml. of saturated $(\text{NH}_4)_2\text{SO}_4$. Allow to stand overnight in icebox. Remove the small amount of precipitate and discard.
- j. Add four volumes of acetone and place in icebox overnight. Collect precipitate by centrifugation.
- k. Dissolve precipitate in 133 ml. of water with the aid of 0.1 *N* NaOH, and mix solution with one-half volume of concentrated NH_4OH . Allow to stand for seven hours at room temperature.
- l. Add 1800 ml. acetone. Collect precipitate by centrifugation.
- m. Dissolve precipitate in 75 ml. of water and dialyze until salt free. Any precipitate is discarded.
- n. Adjust to pH 5.4 with 0.1 *N* HCl and remove precipitate.
- o. Adjust solution to pH 4.7. The precipitate is the pure adrenotropic hormone. Wash with acetone three times and dry in vacuo. Yield 400 mg.

The adrenotropic hormone was prepared in pure form by two groups using somewhat different procedures. The final products from each laboratory agreed in the physiological activity and in their physical properties. Both preparations were shown to be pure by electrophoretic and sedimentation criteria.

15. Isolation of anterior hypophyseal growth hormone (153)

- a. Grind fresh ox pituitaries and dry with chilled acetone (-10°).
- b. Extract 400 gm. of the dry powder with 12 liters of $\text{Ca}(\text{OH})_2$ solution (about pH 11.5) for 24 hours. Lower pH to 8.7 with CO_2 gas, allow to stand for 24 hours and centrifuge.
- c. Add solid $(\text{NH}_4)_2\text{SO}_4$ to a concentration of 2 *M*. Collect precipitate by centrifugation, suspend in 4 liters water, and add $(\text{NH}_4)_2\text{SO}_4$ to 0.6 *M*. Remove precipitate and discard.

- d. Add $(\text{NH}_4)_2\text{SO}_4$ to 2.0 *M*. Collect precipitate, dissolve in 1 liter water, and dialyze until salt free. The pH should now be 6.5–7.0. If not, adjust with 1 *M* HCl or NaOH.
- e. Collect insoluble globulin fraction and dissolve in 600 ml. water by addition of 1.0 *M* HCl to pH 4.0. Add NaCl to a concentration of 0.10 *M*. Discard precipitate.
- f. Add solid NaCl to 5.0 *M*. Collect precipitate and redissolve in 300 ml. water as before, and repeat e and f twice.
- g. Dissolve the final precipitate from f in 200 ml. water and dialyze until salt free. Without removing precipitate, adjust to pH 5.7–5.8 and remove any insoluble material by centrifugation.
- h. Adjust solution to pH 8.7–8.8 and again remove precipitate.
- i. Add $(\text{NH}_4)_2\text{SO}_4$ to 1.65 *M* at pH 7.0. The precipitate is collected, dissolved in water, and dialyzed salt free; g, h, and i are then repeated twice.
- j. The salt-free solution is again precipitated at pH 5.7–5.8 and pH 8.7–8.8 as above, and finally at pH 6.8–6.9. The pH 6.8–6.9 precipitate is collected, and the isoelectric precipitation repeated twice.

The final globulin precipitate is the growth hormone. Approximately 40 mg. are obtained from one kilogram of fresh ox pituitary tissue. The growth hormone, isolated by the foregoing procedure, has been shown to be homogeneous by electrophoretic, diffusion, and solubility criteria.

16. Crystallization of hypophyseal growth hormone (276A)

- a. Freeze 300 to 350 gm. of fresh anterior lobes of bovine pituitary glands by mixing with coarsely ground solid carbon dioxide.
- b. Grind to a fine powder by two or three passes through a grinder and allow residual CO_2 to evaporate. Carry out all subsequent operations at 0–5°.
- c. Suspend ground glands in 2 liters dilute $\text{Ca}(\text{OH})_2$ solution, pH 11.5, and stir mechanically for 24 hours. Maintain pH at 11.5 during first 3–4 hours of extraction by adding solid $\text{Ca}(\text{OH})_2$.
- d. Adjust pH of suspension to 8.5–8.7 with CO_2 gas. Allow suspension to settle overnight. Centrifuge and discard precipitate.
- e. Add aqueous alcohol (equal volumes 95% ethyl alcohol and water) dropwise (Ca 60 ml. per hr.) to a concentration of 12% alcohol. Remove precipitate by centrifugation. Dissolve in water and lyophilize = Fraction A.
- f. Add 1:1 alcohol as above to a concentration of 24% while vigorously stirring the solution. Precipitate removed by centrifugation, dissolved in water and lyophilized = Fraction B.
- g. Adjust pH to 6.8 by slow addition of 4 *N* HCl while stirring. Collect precipitate, dissolve in water and lyophilize = Fraction C.

- h. Adjust pH to 4.6 as in g. Lyophilized precipitate = Fraction D.
- i. Increase alcohol concentration of supernatant from h to 40% by dropwise addition of 1:1 alcohol. Collect precipitate by centrifugation. Discard supernatant. Lyophilized precipitate = Fraction E.
- j. Fractions A, B and C contain essentially all the growth hormone. Dissolve any one (or all) in 0.1 *N* KCl to a 0.5% protein solution. Adjust pH to 11.0 with 0.1 *N* KOH. If necessary, remove undissolved material by centrifuging.
- k. Adjust pH to 5.0 with 4 *N* HCl. Remove precipitate by centrifugation, resuspend in $\frac{1}{2}$ original volume of KCl solution and save.
- l. Adjust the pH of the water-clear and nearly colorless solution to 8.5–8.7 with 1 *N* KOH.
- m. Add aqueous ethanol (1:1) dropwise (rate 30 ml. per hr.) to the solution with constant stirring until the ethanol concentration is 5%. The resulting precipitate which is partly crystalline is centrifuged off and saved.
- n. Bring supernatant to ethanol concentration of 20% using technique given in m. A crystalline precipitate forms slowly and steadily during the period of addition. It is pure or essentially pure growth hormone. Collect by centrifugation, suspend in distilled water, dialyze to remove salt and dry by lyophilization.
- o. Precipitate at pH 5(k) and 5% ethanol precipitate (m) may be dissolved in $\frac{1}{2}$ original volume of KCl with the aid of 1 *N* KOH and reworked to yield additional crystalline material.
- p. Approximate yield of crystalline material from fractions A, B and C = 2.99 gm. per kilo fresh glands.

17. Fractionation of beef plasma proteins with alcohol (54)

- a. Dilute chilled (2–5°) fresh citrated blood plasma with an equal volume of 0.9% NaCl.
- b. Introduce into a cellophane membrane sufficient 40% ethyl alcohol (containing 0.9% NaCl) to make the diluted blood plasma 10% with respect to alcohol when dialysis equilibrium has been reached. The cellophane membrane is suspended in the plasma, and rotated by a motor, until equilibrium has been reached.
- c. Remove precipitate by centrifugation at 0°. Wash precipitate with 10% alcohol from cellophane bag and dry immediately by lyophilization. This fraction contains largely fibrin and fibrinogen and small amounts of serum globulin.
- d. Increase the alcohol concentration, using same technique, to 20%. Bring temperature to –5° and bring alcohol concentration to 30%. Collect precipitate by centrifugation at –5° and dry by lyophilization. This fraction contains predominantly

the γ globulins of the blood plasma and comprises about one-fourth of the total proteins.

- e. Bring alcohol concentration to 40% by dialysis against 55% alcohol solution containing sufficient acetic acid-acetate buffer to bring the entire solution to ionic strength 0.05, pH 5.6-5.8. Collect precipitate and dry as before. The precipitate contains mainly α and β globulins.
- f. Adjust pH of 40% ethyl alcohol solution to 4.8 by addition of acetic acid and chill to -15° . The albumin is precipitated and is collected and dried as before.

The fractions of the blood plasma prepared as described must not be considered to be pure components of plasma. They contain mainly one or two components as indicated, but also smaller amounts of other proteins. For further purification, resolution and reprecipitation at the proper alcohol concentration is necessary. For application of the technique to human blood plasma the reader should consult Cohn et al. (56).

18. Preparation of lipovitellin and vitellin from egg yolk (6)⁷

- a. Break out and separate yolks and whites of three dozen fresh eggs.
- b. Emulsify the yolks and dilute with two volumes of water.
- c. Centrifuge in Sharples centrifuge (bowl speed about 40,000 r.p.m.). Supernatant is used for preparation of lipovitellenin.
- d. Emulsify the precipitate from the Sharples bowl in water to a thin paste and dry by lyophilization.
- e. Extract dried material five or six times with two volumes of ethyl ether until ether is colorless. Discard extracts.
- f. Dissolve $\frac{1}{2}$ of the residue in 500 ml. 10% NaCl and centrifuge to remove any insoluble material. Dialyze against running distilled water until salt free.
- g. Collect precipitate by centrifugation and repeat solution in 10% NaCl and precipitate twice. The final precipitate of lipovitellin is dissolved in NaCl solution for storage. If stored in dry form after lyophilization, its solubility gradually decreases.
- h. Extract the second weighed portion of ether extracted lipovitellin (from e) four or five times with 2 or 3 volumes of cold ethyl alcohol. Dry the insoluble vitellin in vacuo and weigh. Note that the alcohol removed from 16 to 18% of the weight of the lipoprotein. Vitellin is a phosphoprotein, contains 1% P, and is soluble in alkaline solution.
- i. Evaporate alcohol solutions and dissolve residue in ether. The phospholipids are now soluble in ether, but ether did not remove them from the lipoprotein complex. Alcohol, however, breaks up the conjugate leaving the protein lipid free.

⁷ Phosphoprotein preparations, which appear to be very similar, containing 9-10% phosphorus, have been prepared from the protein fraction of egg yolk by a number of workers (26a, 148a, 168a). Meeham and Oleott (168a) have termed their preparation "phosvitin" and refer to it as the principal phosphoprotein of egg yolk.

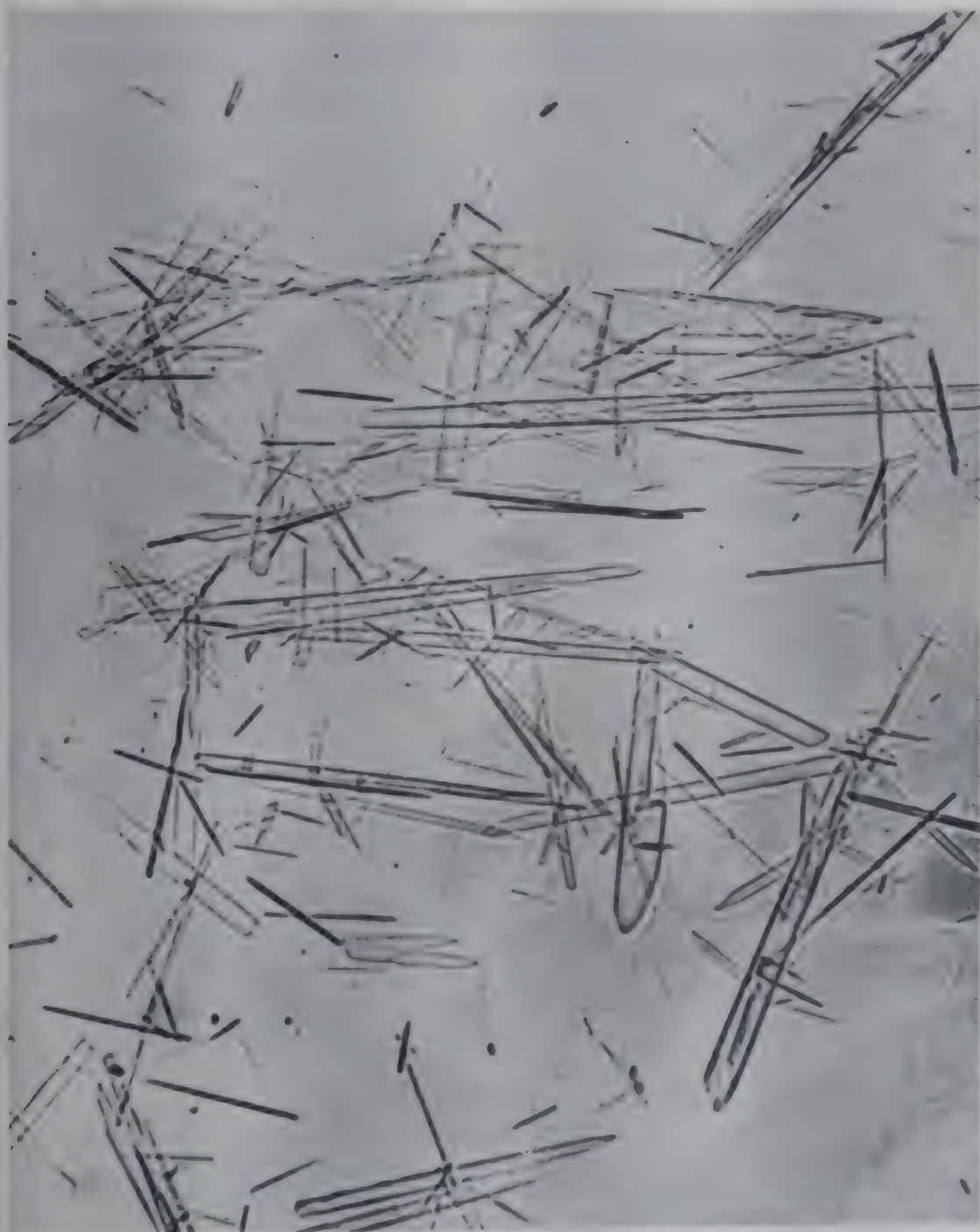


FIG. 8. Pepsin crystals from 15% alcohol. (From Northrop, John H.: *J. Gen. Physiol.*, 30:177, 1946.)

19. Preparation of lipovitellenin and vitellenin from egg yolk (76)

- a. Divide the Sharples supernatant (17-c) into two equal portions in two 2-liter separatory funnels. Add two volumes of ethyl ether and shake. Allow to stand at room temperature for 24 hours, until sharp separation of three layers has taken place.
- b. Draw off the aqueous bottom layer, pour off the ether layer, and discard both. Extract precipitated lipovitellenin five or six times with ether until ether is essentially colorless.

- c. Without removing residual ether, dissolve the lipoprotein in 400 ml. of 10% NaCl solution. Solution should be essentially clear. If not, centrifuge or filter by suction.
- d. Dialyze against distilled water to remove salt.
- e. Centrifuge, and take up in an equal volume of 10% NaCl. Note that the solution remains milky. Add ether to saturation, when solution should become clear. If not, denaturation of the lipoprotein has taken place. Reprecipitate as before. Repeat a third time.
- f. Divide solution in two equal parts and lyophilize one-half and weigh.
- g. Extract weighed portion five or six times with 95% ethyl alcohol. Dry and weigh residue. Lipovitellenin is decomposed by alcohol, as is lipovitellin, with the extraction of 38–40% of phospholipids. Vitellenin, the protein residue, is a phosphoprotein containing 0.28% phosphorus, less than $\frac{1}{3}$ that of vitellin. It is soluble in alkaline solution and precipitates when the solution is acidified.
- h. Dissolve the second half of the lipoprotein after centrifugation in 10% NaCl plus ether. It is stable at room temperature in solution. In dry form it rapidly becomes insoluble.

20. Crystallization of pepsin from alcohol (185a)

- a. Dissolve 100 gms. Cudahy 1/10,000 U.S.P. soluble pepsin in 500 ml. of 20% alcohol. Adjust pH to 3.0 with 10 M H_2SO_4 and allow to stand at 5° for 20 hours.
- b. Add 5 gm. of filter cell and filter.
- c. To the filtrate add 500 ml. saturated MgSO_4 slowly from dropping funnel with stirring and allow to stand 20 hours at room temperature.
- d. Collect precipitate by suction filtration (about 35 gms.).
- e. Stir precipitate with 1 liter 25% ethyl alcohol and adjust pH to 3.8–4.0.
- f. Add 2 gm. filter cell and filter.
- g. Adjust filtrate to pH 1.8–2.0 with 10 M H_2SO_4 . Inoculate with a small amount of crystalline pepsin (needlelike form) and allow to stand at about 20° for two to three days stirring occasionally. A heavy crop of crystalline plates and needles should result. Collect by suction filtration (about 15 gm.).
- h. For recrystallization dissolve 15 gm. of product from (f) in 100 ml. 20% ethyl alcohol and adjust pH to pH 4.0. Add 2 gm. filter cell and filter.
- i. Adjust filtrate to pH 1.8 with 10 M H_2SO_4 and allow to stand one or two days at 20°.
- j. Collect crystalline precipitate by filtration. Yield about 10 gm. The crystals are needlelike in form which is typical of pepsin crystallized from alcohol (Fig. 8). From aqueous solutions pepsin crystallizes in typical hexagonal bipyramids (185).

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Chapter VI

MOLECULAR SIZE OF PROTEINS

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I. INTRODUCTION

THIS CHAPTER details two common supplementary ways of estimating the molecular size of proteins. The theory is indicated, but not rigorously developed. Other useful methods are barely mentioned. The results also give evidence of the relative uniformity of the sample and of the molecular shape.

The data found are of interest because they help characterize protein preparations and afford a basis for interpreting the unique stability of proteins and their reactions among themselves and with other molecules. The molecular size, shape, and homogeneity of proteins is generally affected by such interactions, and, in addition, the configuration of the molecules can also affect their chemical reactions.

In accordance with their morphology, proteins are often classed as *corpuscular* or *fibrous*. The generally soluble corpuscular proteins, including, for example, the protamines, the blood proteins, and the viruses, range in molecular weight from several thousands to many millions and in shape from nearly spherical to extended, rod-shaped forms. On the other hand, the fibrous proteins, including collagen, elastin, and keratins of hair, skin, and wool, are relatively insoluble and possess indefinite molecular size.

Despite wide variation in size and shape, proteins have a common molecular design. Corpuscular and fibrous proteins alike consist of long flexible chains with as many as several hundred amino acid units serially joined through peptide linkages in the manner suggested by Emil Fischer. The number, kind, and distribution of the component amino acids, together with the environment, determine the chemical and physical properties of the protein molecules.

Two extreme shapes may be imagined for any linear flexible molecule: completely extended and compactly folded forms. All evidence indicates that corpuscular proteins consist of one or several chains compactly and regularly folded. The chains are held in specific configurations by attractive forces between the folded segments. The fibrous proteins, on the other hand, consist of indefinite numbers of chains held in relatively extended configurations. In both instances similar forces maintain the specific existing arrangements, which become unstable when the forces are diminished, internal Brownian movement then causing the chains to whip about and assume generally loosely coiled, random configurations.

The common attractive forces responsible for holding protein chains in specific configurations include (a) the hydrogen bond, involving the attraction of a hydrogen atom joined to a nitrogen or oxygen atom for unshared electrons of another oxygen or nitrogen atom; (b) the salt linkage, due to electrostatic interaction of a basic group, such as the ϵ -amino group of a lysine residue, and an acid group, such as the carboxyl group of a glutamic acid residue; and (c) the covalent disulfide bond formed by oxidation of two cysteine residues. In addition to these there are the weaker, so-called van der Waal's attractions involving easily displaced electrons in neighboring atoms. Although many of these bonds are individually weak, their effects in a system of chains are cumulative.

The configuration and stability of a native protein depend on the nature and extent of these interactions, and through them are determined by the environment. When these bonds are broken, for example by shift in pH of the solution or by the presence of specific agents such as urea or detergents, the chains, freed from restraints as either corpuscular or fibrous proteins, will behave similarly as randomly coiled *denatured* proteins.

A conspicuous property of many corpuscular proteins is relative uniformity in size. In contrast to the predictably heterogeneous result of random chemical reaction, many proteins are found with a

remarkably narrow size distribution, evidence of a highly ordered natural synthetic process. It is, moreover, possible that inhomogeneity of some less homogeneous proteins results from the methods of isolation, showing instability rather than fundamental polydispersity of the native protein.

Proteins differ widely in sensitivity toward environment. Variations in pH, total protein concentration, salt concentration, or the presence of other proteins, or special agents, often affect size and shape, and therefore homogeneity through dissociation, unfolding, or aggregation. Many proteins show well-defined pH limits of stability between which each consists of species of similar size and shape, and outside of which it may either dissociate, for example, into halves or eighths, unfold, or associate into units many times larger than the original molecule. The protein hemocyanin from *Helix pomatia* illustrates this behavior (90, page 366; 25, 12).

The presence of specific agents in solution with proteins can also cause dissociation, as of hemoglobin, by urea (13) or of insulin, by sodium dodecyl sulfate (61).

Salt concentration has a marked effect on protein dissociation and unfolding (90, 12, 55, 56).

Finally, in view of the mass action law, it is not surprising to find protein dissociation and association reactions influenced by the concentration of the protein in solution. Thus, hemoglobin and thyroglobulin partly dissociate on dilution (90, page 356; 56). It is probable that many other proteins dissociate likewise, although present methods do not permit study of sufficiently dilute solutions to show striking changes. On the other hand, the tendency for association is already evident in the concentrations, 0.2 to 2.0%, commonly used for molecular size measurement of proteins by osmotic pressure and diffusion and sedimentation methods. This tendency is shown by the deviation of the behavior of the solutions from the ideal; for some proteins noted, particularly for those having elongated shapes, it is necessary to carry out measurements at several concentrations and then to extrapolate the values obtained to zero concentration in order to estimate values independent of interaction effects.

Considerable discussion has existed with regard to the term *molecular weight* as applied to proteins, especially since the measured molecular weight is known to vary with environment. The term "*half-molecule*" implies a definition different from the chemical definition of molecular weight: i.e., a certain standard multiple of

the weight of the smallest possible unit of a substance still having its characteristic chemical properties. Adopting a physical point of view, we can consider the molecular weight to be the weight of the molecular kinetic unit and define molecular weight experimentally as that weight of given substance in solution which, contained in 22.4 liters at 0°, exerts a pressure of 760 mm. of mercury. The commonly used methods for the determination of molecular weight, for example, the methods based on the depression of the freezing point, the elevation of boiling point, or the lowering of vapor pressure of a solution are based on this property. Moreover, the diffusion and sedimentation methods, which are peculiarly suitable for the study of proteins, are also based on molecular kinetic theory.

The molecular kinetic theory assumes that solute molecules in sufficiently dilute solutions behave as an ideal gas confined to a volume equal to the volume of the solution. Because the solute molecules have thermal energy derived from impacts with solvent molecules they are in continual motion and seek to escape confinement. In sufficiently dilute solution the external pressure required to constrain them is related to their molecular weight by the van't Hoff equation:

$$P = \frac{RTc}{M} \quad P/c = \frac{RT}{M} \quad (I)$$

where R is the molar gas constant equal to 8.3×10^7 ergs per degree, T is the absolute temperature, c is the concentration in grams of solute per cubic centimeter of solution, M is the molecular weight, and P is the osmotic pressure in dynes per square centimeter.

Because the ratio of concentration to molecular weight is proportional to the number of molecular kinetic units in the specified volume of solution, the osmotic pressure is a direct function of this number, and the molecular weight, as determined from the osmotic pressure, is an ordinary *number average* molecular weight (72) when material is present having a distribution of molecular sizes. Although the osmotic pressure method applied to proteins gives meaningful results even though the pressures developed are low, the vapor pressure lowering and the depression in freezing point (and the rise in boiling point) caused by proteins are so small, that taken with other considerations the latter methods are not very useful for estimation of molecular size of these substances. The sedimentation and diffusion methods give additional information when the protein is not uniform in size or where several are present. Furthermore, the

sedimentation and diffusion methods are applicable to the determination of the molecular weights of proteins giving osmotic pressures too low for accurate measurement.

It is frequently desirable to obtain the molecular weight by several methods, particularly when the protein is not homogeneous. Because the osmotic pressure method gives a number average molecular weight, while the sedimentation and diffusion methods give *weight average* quantities (as well as other averages) (45, 72), comparison of these average values provides a measure of the degree of inhomogeneity of the protein with respect to molecular weight. For a homogeneous protein the molecular weight determined by the several methods is the same within the experimental error.

These methods all depend on the escaping tendency of molecular kinetic units which, in the ideal case, are free from constraint by intermolecular forces. All of these methods involve the setting up of concentration gradients in solution. Under this condition, the escaping tendency of the molecules is made manifest by the tendency for spontaneous mixing, a tendency which persists until the concentration is uniform throughout the solution.

For osmotic pressure determinations the mixing of the protein is prevented by a membrane impermeable to the protein but permeable to the solvent. Under this condition, the transfer of solvent is the only process which can occur, taking place from the *higher solvent concentration*, in the pure solvent, to *its lower concentration* in the protein solution. The pressure required to prevent the transfer of the solvent is an exact measure of the osmotic pressure of the solute.

For diffusion measurements the solution is permitted to mix with the solvent. The experiment starts with the protein solution separate from the solvent and the rate of mixing is followed either analytically or optically. The diffusion constant, calculated from the rate of mixing, is characteristic of the protein in the solvent used at the given temperature and is a function of its size, shape, and hydration. The diffusion constant is defined as the amount of solute which passes in unit time through a unit cross section under a unit concentration gradient (concentration gradient: the rate of change of concentration with respect to distance), and has the physical dimensions $l^2 t^{-1}$ (length squared divided by time). The diffusion constant is particularly useful when combined with the sedimentation constant to give the molecular weight of the protein.

Two methods are used in sedimentation analysis, both of which

depend on the influence of a centrifugal field in causing the unmixing of a solution in opposition to the mixing process that occurs spontaneously. In one method, the sedimentation velocity method, the sedimentation constant is determined. The sedimentation constant is defined as the sedimentation rate referred to a unit centrifugal field and has the physical dimensions of time. For proteins the values range from 1×10^{-13} to about 150×10^{-13} sec., or expressed in terms of Svedberg units: from 1 to 150 S. A relatively high centrifugal field is employed so that sedimentation of the protein occurs with sufficient speed that the boundary between the solvent and the receding protein does not blur excessively during the time of measurement. Under this condition the rate of sedimentation can be determined. The sedimentation constant is calculated from the rate of sedimentation; like the diffusion constant, it is characteristic of the protein in the solvent used at the temperature of measurement and is a function of size, shape, and hydration of the molecule. For comparative purposes, sedimentation constants are best referred to the standard medium, water at 20° , at the limit of zero concentration (in the absence of dissociation).

In a second sedimentation method, a condition of equilibrium is established between the opposing tendencies of sedimentation and diffusion. A relatively low centrifugal field is employed. When equilibrium is attained, the molecular weight of the protein is calculated directly from its distribution in the cell.

These are not the only methods applicable to the determination of molecular weights of proteins. For example, with adequate knowledge of the amino acid composition of a protein or of its content of some of the more unusual constituents, e.g., sulfur or phosphorus, a minimum molecular weight can be calculated from the analytical data (10, 11). When large crystals of the protein are available and their density is measured, the molecular weight can be determined by x-ray analysis (20, 26, 27). Furthermore, when the protein molecules are sufficiently large to be resolved by the electron microscope a direct measurement of the size and shape can be carried out (81). Additional methods for the determination of molecular weights of proteins have been introduced—one based on surface film measurements (14), another on light scattering (21, 38), and a third on solubility measurements (49). The molecular size of certain proteins has also been determined from dielectric constant dispersion measurements and from streaming birefringence determinations (70, 22).

II. OSMOTIC PRESSURE

The osmotic pressure of a solute is of unique interest because it depends upon the number of dissolved molecules rather than upon their weight or shape. It can, therefore, be used to estimate the molecular weight of a solute in known concentration for which a suitable semipermeable membrane and solvent are available. Second, by comparison of the number-average molecular weight so obtained with average molecular weights found by other methods, e.g., by sedimentation equilibrium in the ultracentrifuge, an estimate can be made of the homogeneity of the solute. Osmotic measurement is the most direct way of estimating molecular weight of a high-molecular substance, such as a protein, in the range 10,000 to 500,000 or even 1,000,000, where the usual physical methods, such as freezing point or vapor pressure lowering are no longer convenient or sufficiently sensitive. By observing the osmotic pressure over a period of time it is possible to determine aggregation or disaggregation. In such experiments the external solvent should be watched particularly carefully for solute and the solution checked for precipitation or other changes. Again, the dependence of osmotic pressure upon concentration, also necessary for accurate molecular weight determinations, can be used as an index of interaction with the solvent (41). In theory, also, the variation of the *reduced osmotic pressure* (P/C) with concentration (C) depends upon the shape of the molecules (98, 99).

Finally, the change in the function $P/C=f(C)$ with temperature permits one to analyze the change in free energy of the solvent due to the addition of solute (proportional to P/C) into heat and entropy terms (temperature dependent and temperature independent) in this way further characterizing the solvent—solute system (60, 28, 42).

1. Apparatus for Osmotic Pressure

Required for the measurement of osmotic pressure are: a membrane selectively impermeable to the solute and a means of measuring the pressure required to prevent net transfer of solvent on one side of the membrane to the solution of protein on the other side. The hydrostatic pressure difference across the membrane necessary to bring the flow of liquid exactly to zero is an exact measure of the osmotic pressure of the solute. An osmometer is an apparatus in which such an equilibrium can be approached and with which the balancing hydrostatic pressure can be estimated.

The simplest osmometers contain the protein solution in a semi-permeable sac joined to a vertical length of capillary tubing and immersed in a vessel containing solvent. Even simple apparatus is able to give meaningful results when used with care (1, 13).

Most workers have modified this apparatus or its use with a view to reaching equilibrium more quickly, improving precision, or using minimum amounts of solution. References to representative specific devices applied to such purposes will now be given.

The first precautions taken are the measurement and control of the temperature either by insulation or automatic temperature regulation. In large apparatus either the solution or the solvent may be stirred. Adjustable counter-pressure may be applied by means of a mercury manometer, a principle recognized and applied, for example, by Sørensen (88). Adair (1) also uses the counter-pressure principle, which has the additional advantages of minimizing change in volume of the solution and of allowing determination of the equilibrium point by interpolation from rates of movement of the capillary meniscus.

Other factors which assist in reaching an equilibrium condition quickly and sensitively include the use of as small as possible a measuring capillary, as permeable as possible a membrane—always subject to the condition that the solute should not appear in the external solvent—and as large as possible a ratio between the area of the membrane and the volume of solution. Some considerations of the latter problems are illustrated by Robertson, *et al.* (83).

To avoid use of the solution as manometric liquid with its tendency to stick to the capillary wall because of the high molecular weight solute present, the manometer may be shifted to the solvent arm as in the apparatus of Oakley (68), which also introduces the use of lighter manometric liquids of low surface tension, of which toluene is popular. Bull and Currie (15) and more elaborately Scatchard, *et al.* (85) have used apparatus derived from Oakley's. Use of a simple osmometer patterned after Bull's is described and illustrated below. Zimm (99) has introduced a small capacity (3 cc.) osmometer in which toluene as a manometric liquid is used in the solution arm and which allows the pressure to be simply adjusted to approximate equilibrium.

A great variety of cells use mechanically supported membranes to minimize change in volume during equilibration. Meyer, *et al.* (60) describe such a cell and illustrate its use (for rubber) in deter-

mining molecular weight and the free energy, heat, and entropy of dilution by principles immediately applicable to other high molecular weight materials including proteins. Other supported membrane cells are described by Carter and Record (16), using a microscope to observe the approach to equilibrium; by Gee, *et al.* (33, 34) who have also been ingenious in developing vapor pressure methods for extending the range of observations beyond the conditions under which direct osmotic measurements are convenient; by Flory (28), by Fuoss, *et al.* (30), and by Goldblum (36), who illustrate the use of the dynamic method of estimating the equilibrium point. None of these workers have been concerned with proteins; but the principles developed are applicable to protein work, subject to appropriate modifications, notably the use of stainless steel instead of brass or corrodible metals in the construction of cell parts exposed to aqueous solutions; in the use of appropriate membrane materials; and in avoidance of decomposition of the protein during the measurements by use of preservatives, low temperatures, short experiments, or sterile technique.

Supported membrane cells of somewhat different types designed specially for use with proteins and using minimum amounts of solution have been described, for example, by Hepp (39) and by Bourdillon (9). Hepp's cell is discussed in English by Peters and Saslow (74) and a simplified form and its use are described by Rehm (82). Bourdillon uses only 0.2 ml. of solution and cites empirical criteria of validity of the measurements.

Jullander and Svedberg (44, 45, 91) and Enoksson (24), exploring means for extending the order of magnitude of reliable osmotic measurements in the region of small pressures, have developed an osmotic cell, the equilibrium pressure in which is determined by weighing. The method is sensitive but requires elaborate precautions, including a constant temperature room.

In all methods, that part of the measured pressure due to capillarity, particularly of the manometric liquid in the capillary tube, must be measured either directly or implicitly, this often being a large part of the total observed pressure. The estimation is often done as a blank experiment, with the same solvent on both sides of the membrane; or the surface tension of the manometric liquids may be measured independently, especially in a capillary of the same diameter, as used in the Zimm cell; or one may simply remove the osmotic contribution to the pressure acting on the manometer (Bull).

2. Details of Measurement of Osmotic Pressure

Parts of an osmometer patterned after one described by Bull are shown in Figure 1 and diagrammed in Figure 2.

To assemble the cell for use, it is first thoroughly cleaned and dried and the stopcock and large ground surface greased lightly. The semipermeable membrane is then prepared. Regenerated cellulose tubing will be found generally suitable as a membrane. Visking 8-32 transfusion tubing fits the apparatus described.

A section of tubing without visible defects is soaked in distilled water until pliable (about 10 minutes or, better, overnight) against the buffer solution. One end is then closed by folding into thirds and tying into a figure-eight knot. This is pulled snug with tension between the knot and the free end, to avoid straining the bag, and the free end is trimmed to about two millimeters from the knot. Such a knot will be protein-tight if made with reasonable care. The



FIG. 1. Photograph of osmotic pressure apparatus after Bull.

bag is then cut to the proper length and carefully worked, wet, over the small ground joint, which it should fit snugly. It is held in place by a rubber or neoprene strip or by a ground glass sleeve matching the joint. A section of rubber tubing may be slipped over the ground joint to accommodate a larger bag.

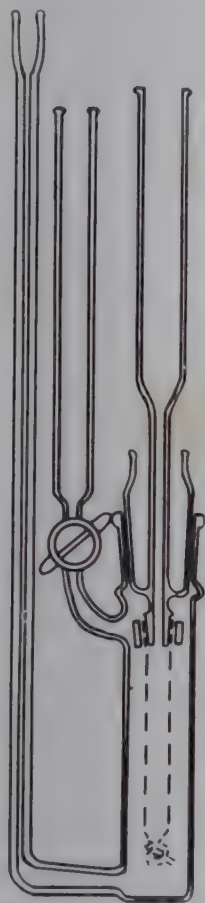


FIG. 2. Diagram of osmotic pressure apparatus after Bull.

The bag is then tested for gross leaks by inflating under buffer and watching for escaping bubbles.

In the meantime a protein solution (say 2%) under study has been dialyzed against a suitable buffer, chosen in accordance with the following considerations:

(a) The protein is reasonably soluble in it giving stable solutions; the protein is not degraded, precipitated progressively, nor aggregated.

(b) It is as near the isoelectric point as practicable and contains salt sufficient to reduce the effect of charge. An ionic strength of 0.1 may be satisfactory (Chap. VII).

(c) When appropriate, attention may be given to selecting a medium which will be suitable for other chemical, physical, or bio-

logical studies. For example, a buffer containing monovalent ions only, of relatively low mobility, may be suitable for electrophoretic study (see Chap. VIII for some examples). If the concentration of the protein is to be determined by nitrogen analysis, a buffer free of nitrogen is desirable.

(d) In an extended, careful study, conditions (a) and (b) need to be verified either experimentally—by testing buffers at several pH's and with various ion species present—or by evidence from other sources. For example, the pH stability range of an enzyme preparation may be independently defined by measurement of enzyme activity.

In assembling the cell, enough manometric liquid is first added to the capillary at the bottom of the cell that the interface with the buffer will stand well within the uniform wide part of the arm joining the capillary. Toluene is commonly used for the manometric liquid. With the stopcock open, buffer, equilibrated against the protein solution, is then added to fill the cell nearly to the top of the ground joint.

The bag is then carefully rinsed with two or three small portions of the protein solution (filtered if necessary) and filled to a level well up (especially for concentrated solutions of low molecular weight proteins where a relatively large osmotic pressure is expected) in the uniform expanded portion of the addition tube. The assembly of the cell is then completed by seating the large, greased, ground joints, excluding air bubbles and forcing buffer up past the stopcock to a level in the uniform expanded portion of its tube somewhat below the level of the protein solution. These levels may now be adjusted as desired by adding or removing buffer or solution as necessary. With experience the condition of equilibrium can be somewhat anticipated, shortening the experiment.

The addition tubes and the top of the manometer tube are covered with dust caps of some sort—for example stoppers with sections of capillary tubing inserted, to reduce evaporation but permit access of air at atmospheric pressure.

The osmometer is then placed in a constant temperature bath in a position such that the liquid levels can be observed with a cathetometer (that is, a precisely mounted reading telescope adjustable along a vertical scale). The temperature may be set conveniently slightly above the maximum expected room temperature; in this instance, extended experiments should be checked for microbial decomposition, or precautions taken to add a preservative (in some cases sodium merthiolate at 0.1 g. per liter may be useful).

The toluene used will help in this respect. If cooling coils are available, the bath temperature may be reduced to the dewpoint, or even below, with double-walled windows. The temperature should not vary over a range of more than 0.05° , and even this may be troublesome. Control to $\pm 0.005^{\circ}$ is about the limit possible without unusual precautions.

Even this variation may be observed experimentally as a variation of height of the toluene column during the heating cycle.

The manometer capillary should be placed approximately vertically, but the exact position is not important since the cathetometer is adjusted to read vertical distances.

In 30 minutes, when the osmometer and its contents have come to temperature equilibrium, cathetometer readings are recorded for the top of the toluene column, of the buffer, and of some convenient fixed reference point on the cell, for example, the upper edge of the stopcock. The experiment is then begun by closing the stopcock, and readings are made periodically at recorded times of the top of the toluene column, of the protein solution, and of the fixed reference point.

There are several methods applicable to finding the equilibrium height of the toluene column.

(a) Wait until the variation in height over a heating cycle is greater than the variation over a "long" (24 hour) period of observation.

(b) With a sufficiently permeable membrane, protein solution may be added or removed to keep the toluene level at or near its initial position. In this way the effect of transfer of buffer across the membrane, changing the concentration of the solution, is minimized.

(c) The direct method (a) may be seriously in error. The field of error can be restricted by approaching equilibrium first with a rising toluene column, then with a falling toluene column.

(d) Successive net hydrostatic pressures, computed from the liquid levels, plotted against the reciprocal of elapsed time often, after a few readings, give a straight line which can be extrapolated to infinite time and so define the equilibrium height. Graph paper may be had with reciprocal, or hyperbolic ruling which is convenient for making such plots without the necessity of taking reciprocals explicitly (Keuffel and Esser No. 358-25).

Sample data and calculations are given in Table I.

In an extended study, particularly in which membranes of modified permeability are used, a measure of the permeability is desir-

able and can be had by following the rate of change of height in the capillary in response to definite applied pressures; for example, by making a pneumatically tight connection to the top of the tube on which the bag is mounted from a mercury manometer with an adjustable levelling bulb. The mercury level will also respond to temperature variation and to stretching of an unsupported membrane.

When the equilibrium height has been determined and the height

TABLE I

SAMPLE DATA AND COMPUTATION OF MOLECULAR WEIGHT
FROM OSMOTIC PRESSURE MEASUREMENT

(A) *Data for the osmotic pressure of a reduced
feather keratin preparation*

Solvent: pH 8.6, ionic strength 0.1 buffer containing 0.1 *N* sodium barbital, 0.02 *N* barbital, diluted with one-ninth its volume of isopropyl alcohol.

Density of solvent: 0.9934 gram per cm.³, from Westphal balance reading at 25°.

Protein concentration: 0.756 mg. protein nitrogen per cm.³ equivalent to 0.48 g. protein, dried at 110°, 24 hours at atmospheric pressure. The concentration was determined from the precipitate insoluble in 10% trichloroacetic acid at the end of the experiment. There was no protein in the dialysate by the same test.

Osmotic Pressure Data							
Date	Hour	Temper- ature°	Cathetometer Readings (Centimeters)				
			Toluene	(Toluene- Buffer Interface)	Solution	Buffer	Refer- ence Mark
<i>Series one</i>							
11-13	17:00		(Closed stopcock to begin the experiment)				
11-14	9:45	24.90	85.050	58.695	81.995	79.530	74.790
11-14	12:00	24.94	84.920	58.690	82.000	79.505	74.785
11-14	17:00	24.92	84.590	58.670	82.000	79.525	74.775
11-15	8:45	24.92	83.570	58.650	81.975	79.520	74.770
11-18	9:00	24.94	79.660	58.555	81.885	79.420	74.800
11-18	17:00	24.94	79.445	58.560	81.905	79.435	74.820
11-19	9:00	24.89	79.100	58.550	81.875	79.410	74.800
The stopcock was then opened.							
11-19	10:15		84.330	58.685	81.890	79.325	74.780
<i>Series two</i>							
11-19	10:45		(The toluene level was forced below the equilibrium position and the stopcock closed)				
11-19	11:00	24.94	73.975	58.475	81.835	79.480	74.850
11-19	17:15	24.92	75.100				74.875
11-20	9:15	24.90	76.500	58.610	81.838	79.523	74.920
11-20	16:30	24.95	76.765	58.585	81.810	79.465	74.865
11-21	9:00	24.92	77.140	58.580	81.780	79.435	74.885
11-21	17:00	24.92	77.245	58.595	81.765	79.435	74.880
11-22	9:00	24.91	77.350	58.600	81.730	79.415	74.885
11-25	9:00	24.94	77.540	58.620	81.660	79.365	74.880
11-25	11:15	24.93	77.540	58.615	81.645	79.340	74.880
The stopcock was then opened.							
11-25			84.180	58.775	81.680	79.265	74.840

TABLE I—*Continued**(B) Notes on the osmotic pressure data*

The cathetometer readings of the toluene-buffer interface and, except for the italicized values, of the surfaces of the solution and the buffer are unnecessary for routine computation of osmotic pressure, as given, but are included in the table to illustrate the following features.

The simplified computation used supposes that only a negligible error is made by assuming a stationary toluene-buffer interface. The data show that, during the measurements, the interface approaches a position about 0.20 cm. below its equilibrium position with the stopcock open. This means that the computed osmotic pressure will be small by 0.2 times the difference in density between the toluene and buffer. This error amounts to 0.03 cm. of water (grams per square centimeter), or about 1 % in this instance, and might be worth taking into account.

The level of the solution is seen to have fallen by 0.1 and 0.2 cm. in the respective series. This movement must be accounted for as the resultant of liquid transfer through the membrane and evaporation. Inasmuch as the buffer level fell by about 0.1 cm. in both series, the movement of the solution level in the first series was due largely to evaporation. In the second series, transfer of the liquid through the membrane from solution to solvent accounted for about as much fall in the solution level as did evaporation. This circumstance may account for the difference in the mode of approach to equilibrium shown by the graphs (part C, this Table).

(C) Graph of the net difference in toluene level with the stopcock open or closed as a function of time plotted on reciprocal ruled coordinate paper.

The equilibrium value for the second series of readings by extrapolation is 6.45 centimeters

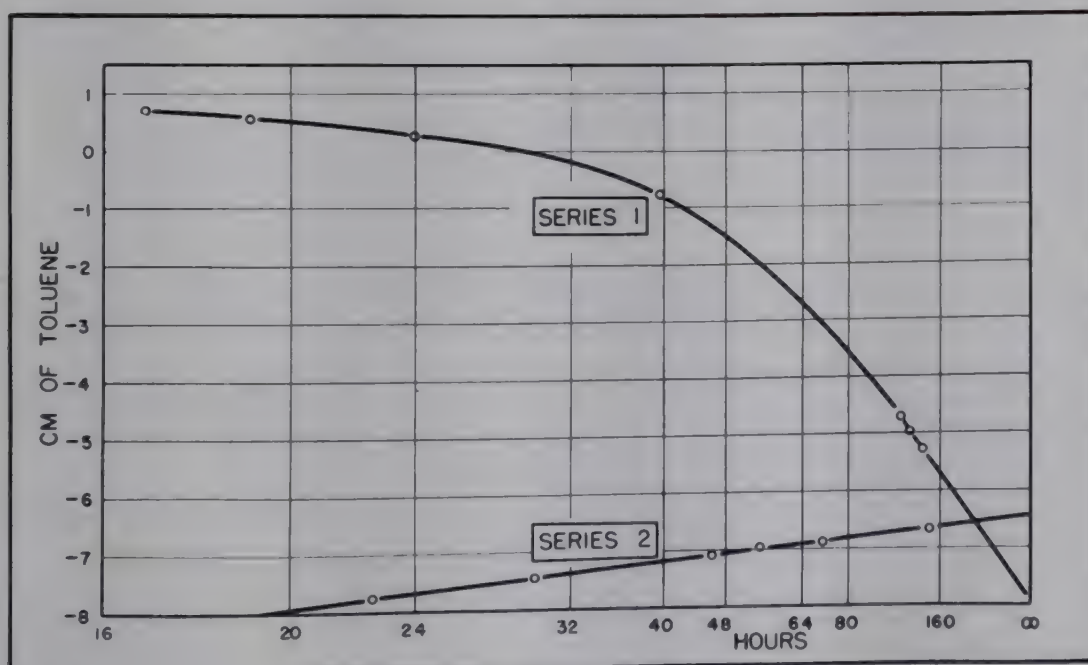


TABLE I—Continued

(D) Computation of osmotic pressure and molecular weight for the second series: (1) with the final readings taken as the equilibrium value, and (2) using a graphically extrapolated value for the net difference in toluene level

Final toluene reading: cm.	77.540	
Reference point	74.880	
Difference	2.660	
Toluene reading with open stopcock	84.180	
Reference point	74.840	
Difference	9.340	
Net difference in toluene level	(1) 6.680	(2) 6.45
Density of toluene	0.862 g. cm. ⁻³	
Net difference in toluene level: in units of cm. water (unit density) or gm. cm. ⁻²	(1) 5.758	(2) 5.56
Final solution level: cm.	81.645	
Reference point	74.880	
Difference	6.765	
Buffer level with open stopcock	79.265	
Reference point	74.840	
Difference	4.425	
Net difference in buffer-solution levels	2.340	
Density of buffer: g. cm. ⁻³	0.9934	
Net difference in buffer-solution levels: cm. water (unit density)	2.32	
Osmotic pressure, π : cm. water	(1) 8.083	(2) 7.88
Protein concentration, $c = 0.4797$ g. per 100 cm. ³		
Reduced osmotic pressure, π/C : cm. water (unit density) per g. of protein per hundred cm. ³	(1) 16.85	(2) 16.43
Molecular weight, M : from the relation: $M = 252,750 c/\pi$.	(1) 15,000	(2) 15,386

(E) Notes on the evaluation of the molecular weight

The van't Hoff formula $PV = nRT$ and its various forms: $M = mRT/PV$, where $n = m/M$, and $M = cRT/P$, where $c = m/V$, may have its factors expressed in any self-consistent set of units. In the conventional centimeter-gram-second system,

P , the osmotic pressure, is expressed in dyne cm.⁻² and

V , the volume of the system considered, in cm.³

n , the number of moles of solute in the volume V , is also the weight m grams in the same volume divided by the molecular weight M .

c , the concentration of the solute in g. per cubic centimeter, is m/V .

R , the molar gas constant, has the value $8.3143 \cdot 10^7$ erg degree⁻¹.

T , the absolute temperature, 273.18 plus the centigrade temperature, in series two equals 298.11°.

In the estimation of molecular weight by osmotic pressure, it is convenient to use a larger unit of pressure, centimeters of water, that is, g. cm.⁻², and a smaller unit of concentration, g. per 100 cc. Letting π cm. water express the osmotic pressure and

C grams per hundred cubic centimeters express the concentration, we apply the following conversion formulas:

$$P = 980.64\pi$$

and

$$c = 0.01 C$$

TABLE I—*Continued**(E) Notes on the evaluation of the molecular weight—continued*

and derive:

$$M = \frac{0.01 \cdot C \cdot 8.3143 \cdot 10^7 \cdot 298.11}{980.64\pi} = 252,750C/\pi.$$

A single osmotic pressure experiment, or several made near the same concentration should not be trusted to give a reliable estimate of the molecular weight in the absence of information of the effect of concentration on the activity of the solute. Whenever practicable, measurements covering several percent of concentration should be made in order to determine the limiting value of the reduced osmotic pressure, π/C , at zero concentration.

of the protein solution measured, the stopcock is again opened and the heights of the toluene and buffer redetermined. The densities of the buffer and of the manometric liquid at the temperature of measurement are determined, and from these data the osmotic pressure is estimated as follows:

Let t_0 cm. be the height of the toluene column, stopcock open,

b_0 cm. be the height of the buffer column, stopcock open,

r_0 cm. be the height of the reference point, stopcock open,

t cm. be the height of the toluene column, near equilibrium, stopcock closed,

p cm. be the height of the protein solution, near equilibrium, stopcock closed,

r cm. be the corresponding height of the reference point as measured near equilibrium,

d_t g. cm.⁻³ be the density of the toluene,

d_b g. cm.⁻³ be the density of the buffer.

Osmotic pressure, π cm. water at 4°, is approximately

$$(p - r - b_0 + r_0)d_b + (t_0 - r_0 - t + r)d_t$$

or in case

$$r_0 = r, \quad \pi = (p - b_0)d_b + (t_0 - t)d_t.$$

The equilibrium value may now be reapproached from a different initial value, for a check, or the solution and buffer may be removed for analysis. The validity of this equation depends upon:

- (a) The difference in capillarity of the protein solution and buffer being of a negligible order of magnitude in the wide addition tubes;
- (b) The difference in densities of the protein solution and buffer being negligible;
- (c) The change in height of the toluene-buffer interface being negligible.

All of these data enter explicitly into the exact computation of the osmotic pressure.

3. Computation of Osmotic Pressure and Molecular Weight

The osmotic pressure in a sufficiently dilute solution of known concentration may be used to estimate the molecular weight by means of the van't Hoff formula in the form: $\pi/C = RT/M$, in which π is the osmotic pressure in gram per square centimeter, C is the concentration of solute not diffusing through the membrane in grams solute per 100 ml. of solution, M is the molecular weight, R is the gas constant, having in this instance the value 8,480,000 hundred-gram-centimeters per mole per degree K., and T is the temperature in degrees Kelvin (absolute).

The concentration of the non-diffusing portion of the protein in solution must be known as accurately as the molecular weight is desired. It may be taken as the value to which the original solution was made up, especially with methods in which the transfer of liquid across the membrane is minimized. It may be estimated directly analytically by evaporating (say 110°, in vacuo, 18 hours) measured volumes of solution and solvent and subtracting the weight of the residues. It may be estimated indirectly analytically by comparing the refractive indexes or Kjeldahl nitrogen values of the solution and solvent; or colorimetrically, directly with a colored protein, or with the use of a color reagent for some specific group in the protein: for example, the Folin-Ciocalteu reagent (29). The protein from a known volume may be precipitated by trichloroacetic acid to a final concentration of 10% in a centrifuge tube of a design also adapted for Kjeldahl digestion, resuspended, washed, and analyzed. Indirect methods require standardization against the sample actually used. In this connection one encounters the problem of the somewhat arbitrary character of the dry weight of a protein. As a consequence, the drying conditions applied to the sample may well be recorded, not only to define the moisture content but also in case the protein is altered by the drying process.

Solutions of even moderate concentrations of high molecular substances often show osmotic pressures considerably less than those given by the van't Hoff formula, which is really a limiting law for "infinitely dilute" solutions. This situation is met by measuring the ratio π/C at five or six different concentrations. In many instances π/C will be found to be nearly a linear function of C and the π/C intercept may be taken for computing the molecular weight. The slope of the line is a measure of solvent-solute interac-

tion. Ideal solvents which do not interact with the solute and in which the solute is often not very soluble give lines with small slopes; solvating solvents give lines with larger slopes. Linear dependence of π/C on C may also result from asymmetry of the solute particles (79). Deviations from linearity also have significance. Schulz relates the effects of concentration on osmotic pressure with the effects on diffusion and sedimentation constants in order to eliminate uncertain extrapolation to zero concentration in the determination of molecular weight (86).

III. DIFFUSION ANALYSIS

1. The Purpose of Diffusion Measurements

The principal object in diffusion analysis as applied to proteins is the determination of the diffusion coefficient or diffusion "constant." For spherical, unhydrated molecules the measured diffusion constant is simply related to the molecular radius and molecular weight. Because proteins in general are hydrated and are not necessarily spherical, additional data are required for precise computation of the molecular weight. In the section on sedimentation analysis we shall illustrate how the diffusion constant and the sedimentation constant are used together to determine the anhydrous (that is, as dried under standard conditions) molecular weight of a protein, independent of its shape and degree of hydration in solution. Another aim of diffusion analysis is the determination of homogeneity. The refractometric methods discussed later are uniquely suited to this purpose. Further information may be derived from the dependence of the diffusion constant upon concentration, asymmetric protein molecules showing especially large concentration effects. Finally, a series of experiments can be planned to define the stability limits of a protein in media of varied composition or to demonstrate reactions leading to changes in shape or molecular weight, by means of the effect upon the diffusion constant.

2. The Nature of Diffusion

The net result of diffusion is transfer of material from a region of higher concentration (more rigorously, *activity*) to one of lower concentration. From a macroscopic viewpoint, the process is due to the difference of chemical potential or of osmotic pressure of the diffusing substance between the regions of different concentration. From a submicroscopic viewpoint, however, diffusion results from the thermal or Brownian movement of all particles; the laws

describing diffusion have also been deduced from kinetic theory (23).

The diffusion coefficient, which is dependent upon temperature and upon the viscosity of the medium in which diffusion takes place, is the proportionality factor relating the rate of transfer of material across unit cross-section, to the rate of change of concentration with respect to distance (the concentration gradient) at the given cross-section. In the centimeter-gram-second system, it has the specific value: the number of grams of material transported per second across an area of one square centimeter perpendicular to which there is a gradient of one gram per cubic centimeter per centimeter. This reduces to the unit: centimeters squared per second. It is therefore apparent that the unit in which mass is expressed is unimportant and might be the molecule without affecting the numerical value of the coefficient. As a result of deduction from kinetic theory the diffusion coefficient may also be stated as one-half the average squared displacement in a given direction experienced by a particle in unit time. For a protein molecule, the order of magnitude is about 10^{-6} to 10^{-7} square centimeters per second.

Although the diffusion coefficient is dependent upon the temperature, and especially upon the viscosity of the medium in which the process occurs, the dependence upon these factors is known with sufficient accuracy to make useful the transformation of measured values to standard reference conditions, such as 20° in distilled water, to give diffusion "constants" characterizing the material studied. In fact, the diffusion constant often depends upon the concentration of the diffusing substance, so that the limiting value approached at lowest concentrations should be cited as the constant. The terms *coefficient* and *constant* are often used as synonyms.

3. Principles of Measurement of Diffusion

Since it is not possible to follow the movement of individual protein molecules, measurement of diffusion of proteins, as of other substances, depends upon setting up a sufficiently well-defined concentration gradient and devising a way of measuring the resulting transfer of material with time. The concept of the concentration gradient is useful in ultracentrifugal analysis as well as in diffusion analysis and will be explained in detail. A column of solution is illustrated in diagram A, Fig. 3, in which the circles represent protein molecules. Most of them are near the bottom of the col-

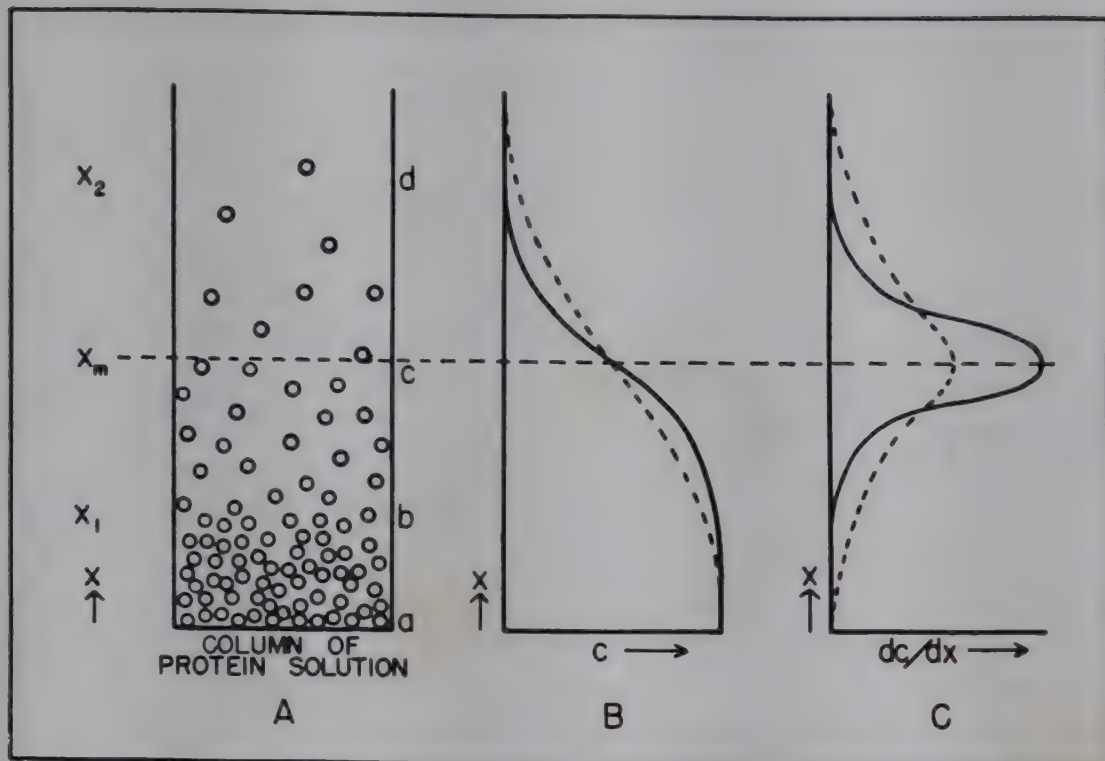


FIG. 3. Distribution of a dissolved substance at a boundary. A. Column of protein solution. B. Variation of concentration with distance. C. Variation of concentration gradient with distance.

umn, where the concentration has a certain value which remains the same from the bottom to the level x_1 , where the molecules begin to thin out noticeably. Above x_2 there are none at all. This state of affairs is represented graphically in diagram B, in which the concentration, or number of grams of protein per ml. of solution is plotted against x , the position in the column. This is the sort of curve given by the light absorption method (see below).

The region between x_1 and x_2 , in which the concentration changes with position in the column is called the boundary. In order to specify the position of the boundary by a single measurement, the value x_m may be selected at which the concentration changes most rapidly. This (modal) position is usually easy to determine. It is theoretically less useful than either the position at which the concentration has half of its maximum value or the position such that the number of molecules above it would be just enough to restore the solution below that level to the concentration of the solution at the bottom of the column. For symmetrical boundaries, these positions coincide.

The modal value, x_m , is shown more clearly, together with other features of the boundary if, as in diagram C, the *rate of change of concentration with respect to distance* rather than the concentration

is plotted against the position in the column. This rate of change, dc/dx , is the concentration gradient at a given position, x . Diagram C may be derived from diagram B by plotting values of the *slope* of the curve in diagram B against the corresponding position in the column; the refractometric methods of analysis (see below) give curves analogous to that of diagram C directly.

Sometimes the word *gradient* is used as a synonym for *boundary*, especially when it is desired to call attention to the boundary as a region of concentration change, in which the gradient has a definite set of values depending upon the position, from which useful information may be derived.

In the course of diffusion some of the molecules of the system illustrated in diagram A will move farther up the column and the concentration will also begin to change at a level nearer the bottom. This result may be precisely illustrated for an ideal system by the dashed lines in diagrams B and C. The S-shaped curve of B is pulled out symmetrically, with the midpoint, where the concentration is one-half its maximum value and where the gradient is greatest, remaining at the same height in the column. The peak in C is flattened out, but the area between the curve and the base line at the side remains the same. In diagram C, the movement of the curve in the direction of the x -axis exactly parallels the molecular movement of protein in diagram A. This means that the average squared distance of the curve C from the mean position x_m changes in a given time interval by the same amount as the average squared displacement of a protein molecule in A in the same direction. The diffusion constant has already been defined as one-half this rate of change. The use of this property will be described in detail in a later section in computing a diffusion constant. The sedimentation constant is determined from the change in the mean position of such a boundary.

In mathematical notation it suffices to point out that to calculate diffusion constants, an integral solution is sought of the equation

$$\frac{\partial c}{\partial t} = \frac{\partial}{\partial x} \left(D \frac{\partial c}{\partial x} \right). \quad (\text{II})$$

Here c is the concentration, t the time, and x the distance in the direction of diffusion.

To integrate this equation it is assumed that c is a function only of $y = x\sqrt{t}$. As shown by Boltzmann, this leads to the form

$$\frac{d}{dx} \left(D \frac{dc}{dy} \right) = -\frac{y}{2} \frac{dc}{dx}, \quad (\text{III})$$

which yields the integral equation

$$D = -\frac{1}{2} \frac{dy}{dc} \int_0^c y dc. \quad (\text{IV})$$

From the Boltzmann equation, D is computed by graphical or numerical integration.

The student is referred to the symposium on *The Diffusion of Electrolytes and Macromolecules in Solution*, for a survey of the problems arising in measurement of diffusion, and of apparatus now in use (65). A review by Neurath discusses the diffusion of proteins in particular (62). These papers will also serve as an introduction to the literature.

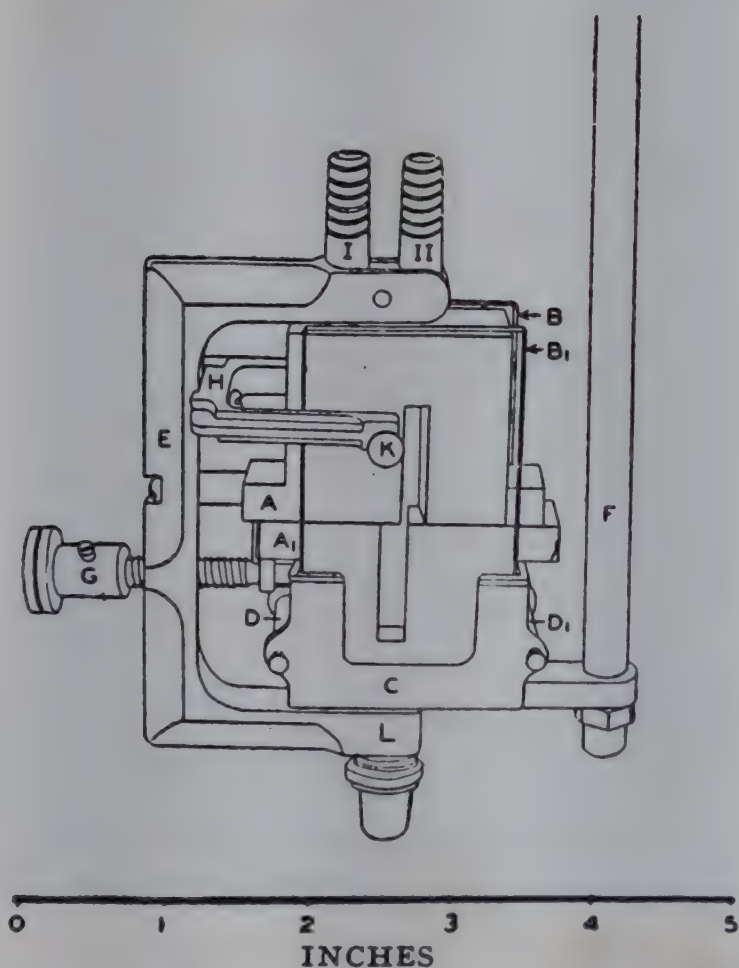


FIG. 4. Neurath-Stamm cell.

4. Apparatus in Which Diffusion is Measured

The apparatus may be classified by the method used to set up the concentration gradient. The most useful method of boundary formation with proteins is probably that using a sliding joint for superposing the solvent on the solution, as in the apparatus of

Cohen and Bruins (18) and Polson (80), the apparatus of Loughborough and Stamm (54) modified by Neurath (63), Fig. 4, or the Tiselius electrophoresis cell (84, 50), Fig. 5, described later in detail. Claesson (17), Fig. 6, has designed a similar sliding joint cell for optical analysis.



FIG. 5. Tiselius electrophoresis cell.

Another useful device, removal of a sliding partition between solvent and solution to form the boundary, is used by Furth (31, 32) in ingenious small instruments designed to shorten the time of an experiment, and by Lamm (48), Fig. 7.

The protein solution may be introduced beneath the solvent through a capillary stopcock as in the cell of Svedberg (92, 89),

Fig. 8; or the solvent may be introduced from above through a sintered glass partition (47).

It is also possible to have the diffusion gradient stabilized within a porous partition to minimize convective disturbances (2, 32a, 37, 40, 57, 67). In this instance the experimenter gets only a single datum from a given experiment.

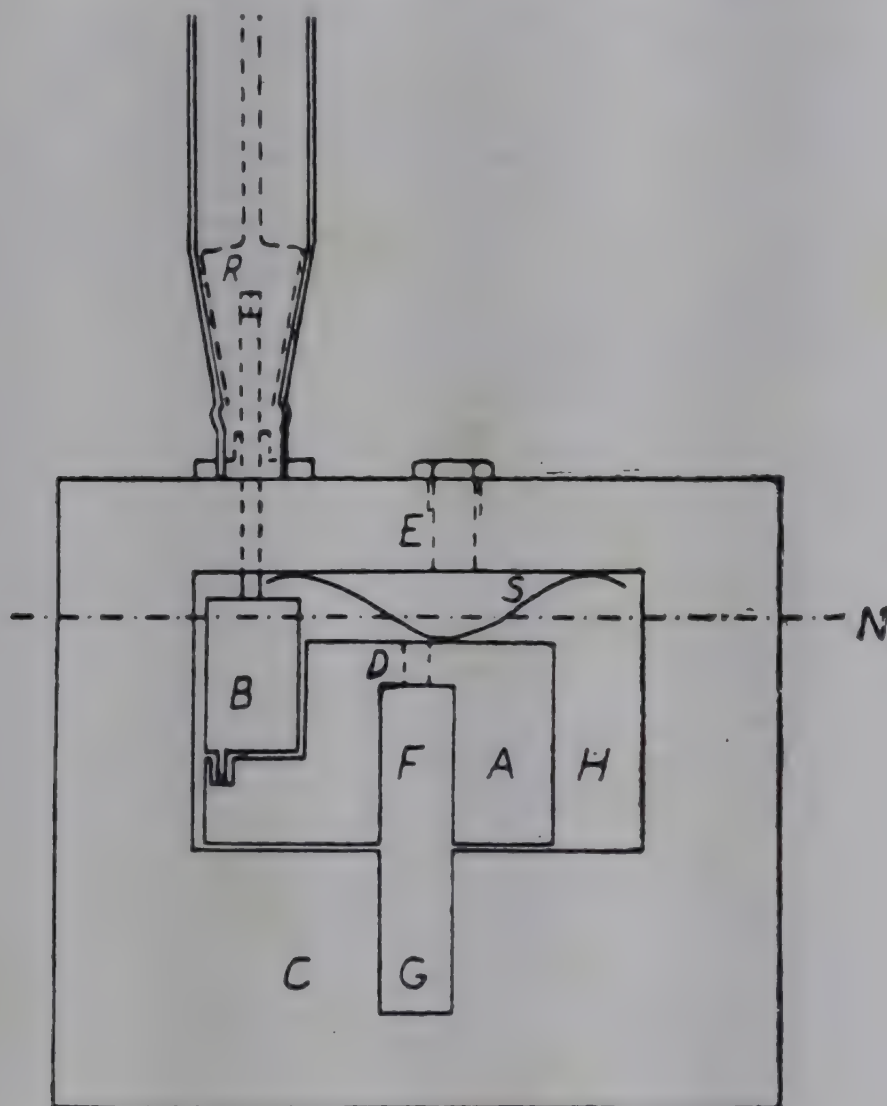


FIG. 6. Claesson diffusion cell.

5. Methods for Measuring Transfer of Material

The actual movement of diffusing material is measured in two general ways. The first consists of analysis of samples removed from the diffusing system at the end of a measured time. In the second, a way is found, usually by optical means, of following progressive changes without disturbing the boundary.

The first, *analytical*, method is experimentally simpler. It is

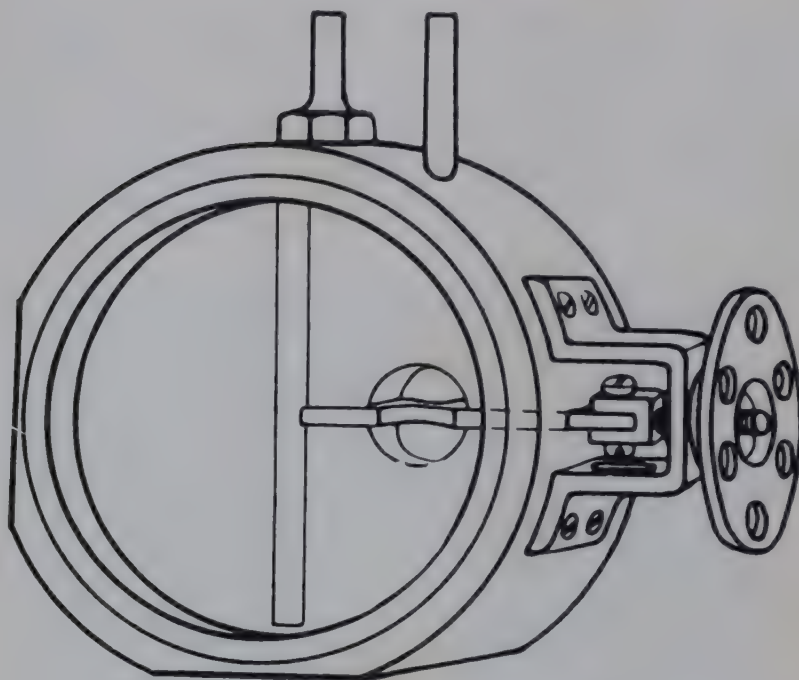


FIG. 7. Lamm-Polson diffusion cell.

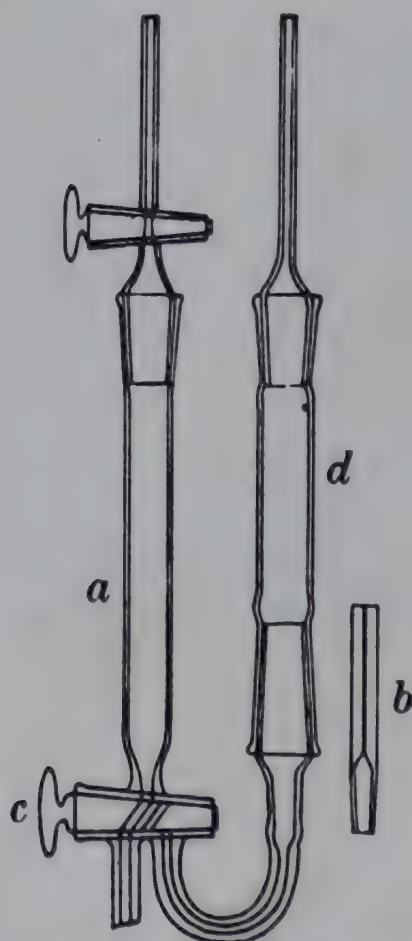


FIG. 8. Svedberg diffusion cell.

applied to the porous partition cells (2, 32a, 37, 40, 57, 67) and to some types of sliding joint or sheared boundary cells (18, 80) to which it is adapted. Experiments in porous partition cells are always comparative, requiring calibration with a standard substance. Calibration with a substance of known diffusion constant is recommended with any method.

The most favorable methods of collecting data are optical. These allow the most detailed study of the actual form of the gradient during an extended period without mechanical disturbance. The data may make use of light absorption (90); the displacement of scale lines seen through the gradient (48); the deflection of a ray of light passing through various parts of the gradient, that is, the so-called *schlieren* method, of which one is given in detail; or interference (46, 53a, 69). In these methods the diffusing gradient is set up in a transparent column guarded against mechanical disturbance and temperature change.

The use of light absorption to determine the form of a concentration gradient was one of the first optical methods to be successfully applied to proteins. It depends upon a linear relation between the amount of light absorbed and the concentration. In it the diffusing column including the gradient is illumined as uniformly as possible by light of a suitable wave-length and photographed at a measured enlargement. Ultraviolet light is used for colorless proteins, with the necessary quartz optical system. The blackening of the photographic plate is converted to a concentration curve of the form illustrated in Fig. 3, diagram B.

In spite of the more complex apparatus required, the scale displacement and *schlieren* methods, based upon refraction of light, have replaced the absorption method. In these refractometric methods a beam of visible light is passed through the column of solution. At any point in the cell where there is a concentration gradient the light is refracted. The extent of refraction is in direct proportion to the magnitude of the gradient at the given point. Several methods are used to record the refraction of light. The most commonly used for sedimentation and diffusion measurements are the scale method (48) and the cylindrical lens *schlieren* method (94, 95, 51). Another, the mechanical scanning *schlieren* method (51) is used widely for measurement of gradients in electrophoresis and can be used also for diffusion and sedimentation when the gradients are not changing too rapidly. The principal advantage of the refractometric methods over the absorption method is that

they are readily applied to the majority of proteins, which are not colored.

The scale method is generally used when the most precise measurements of the concentration gradient are required, as in diffusion or sedimentation equilibrium experiments. This method, however, requires much time and labor for computing and is therefore often replaced by the schlieren methods, which also depend upon light refraction by concentration gradients. The schlieren methods, because they show the form of the boundary more immediately, are particularly suited for sedimentation velocity measurements, for which the rate of sedimentation can be measured directly from the successive positions of the peak recorded on the photographic plate. The scale method employs a transparent linear scale having 25 to 100 lines to the centimeter. The column containing the diffusing boundary is placed between the illuminated scale, or its projected image, and the camera objective. When the camera is focussed on the scale, the presence of a gradient at any region in the cell is detected by the displacement of scale lines seen through that region. The displacement of the scale lines from their normal position is determined accurately to 1 micron using a traveling microscope, or comparator. The displacement, termed the "Z"-value, is plotted as ordinate against the reading of the corresponding scale line to give the graph of the gradient as a function of position in the cell. Although the recorded gradient is the refractive index gradient it is usually unnecessary to convert to the concentration gradient. The specific refractive increment, usually of the order of 180×10^{-5} per gram per 100 ml. is, however, the constant relating the refractive index of a protein solution to the amount of protein in solution; since the photographic record is enlarged by the optical system it is necessary to convert to displacements and distances in the cell by using the appropriate enlargement factor. The first schlieren method (94, 95, 51), Fig. 9, employs a horizontal slit at the light source, a so-called schlieren lens, an adjustable oblique ("diagonal") slit, and a cylindrical lens, in addition to the light source-camera objective-photographic plate system. In operation, an image of the slit at the light source is brought into focus by the schlieren lens through the column of solution in the plane of the diagonal slit. Vertical elements of the column of solution are focussed on the camera plate by the camera objective and cylindrical lens, the latter with its cylindrical axis in the vertical position. Horizontal elements of the diagonal slit are focussed by the cylindrical lens (and in some arrangements, also through the camera objective)

on the photographic plate. In this arrangement the diagonal slit serves as a mask permitting only unrefracted rays of light to strike the center of the cylindrical lens where they are not affected by this lens. Other light rays which are refracted by the concentration gradient in the column of solution, strike the diagonal slit off center and are bent into focus by the cylindrical lens to a position which is correspondingly further to one side of the base line determined by the unrefracted rays. The photographic record is essentially a

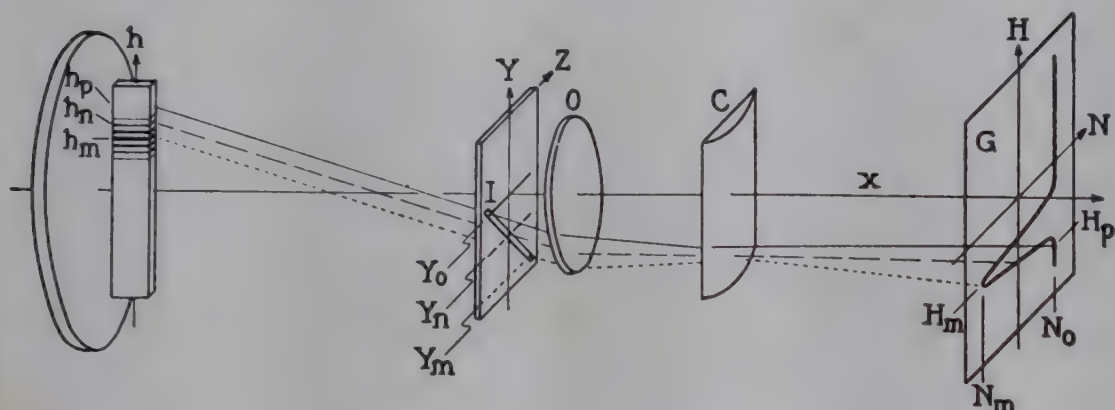


FIG. 9. Illustration of spatial relations in cylindrical lens schlieren method.

graph of the refractive index gradient as a function of position in the cell as in Fig. 3, diagram C. The dimensions of the record depend on the position of the lenses with respect to the other components of the optical system and also on the angle of the diagonal slit, but the degree of enlargement in the direction parallel to the base line is independent of the slit angle. After appropriate conversion of the distances on the camera plate to distances in the column of solution the distribution of concentration in the cell is obtained through the usually valid assumption of proportionality between the concentration of the solution and the increment in its refractive index due to a dissolved substance.

The mechanical scanning system (51) differs from the cylindrical lens method in the use of a vertically movable horizontal blade in place of the diagonal slit and in the absence of a cylindrical lens. The camera plate is moved horizontally at the same time as the horizontal blade is moved vertically. The column of solution is focused by the objective on the camera plate. In scanning, the rays deflected by the gradient are then progressively cut off by raising the horizontal blade making a dark area, growing in size, at the corresponding position of the image. The pattern that is recorded by simultaneous movement of the photographic plate is analogous to

that obtained by the cylindrical lens method and is interpreted in the same way.

6. Adjustment of the Optical System (for the Cylindrical Lens Schlieren Method)

Detailed discussions of the adjustment of the optical systems are given by Svensson (94), Lamm (48), and Longworth (51); however, some details are given here for the cylindrical lens method which may help in setting up the apparatus and keeping it in adjustment (see Fig. 9).

1. Place the cell in the thermostat as near as possible to the window away from the camera plate. Center it with the window. Mark its position so that it can be replaced *exactly* in later experiments. Focussing may be done conveniently with an object (marked horizontal lines on a silvered or blackened strip) mounted in the cell at the prescribed distance from the inner surface of the cell nearer the light source: $a/\sqrt{3}$, where a is the thickness of the column of liquid in the direction of the path of light (94). Focussing can also be done approximately with a boundary gradient in the cell. Light not passing through the cell should be cut off by a mask.

2. The schlieren lens is mounted as near the cell as possible and centered with it.

3. The camera objective is placed with its center at the level of the middle of the cell and schlieren lens. It and the plate holder, with ground glass, are placed to make a sharp image of the object in the cell at the desired enlargement (0.5 to 2.0 times). The positions may be computed to a good approximation from the focal length F_0 of the objective and the relation

$$\frac{1}{P} - \frac{1}{Q} = \frac{1}{F_0}$$

where P and Q are the optical distances (geometrical distance divided by refractive index) from the lens to the object and to its image. The sizes of the object and of its image are in the ratio of these distances.

4. The cylindrical lens is placed with its axis vertical, centered with the optical axis of the system, at least twice its focal length, F_c from the photographic plate.

5. The oblique ("diagonal") slit is placed in its approximate (computed) position so that the cylindrical lens will make a "sharp" image of it on the plate (see step 7). It may be on either side of the objective.

6. The light source and horizontal slit are set up in line with the other components. The lamp is turned with its base so that its metal supports do not obstruct the light; the lamp is raised or lowered in its housing so that the most light possible falls on the schlieren lens. The horizontal slit is moved toward or away from the schlieren lens until its sharp image falls at the position of the oblique slit. The horizontal slit is then raised or lowered until its image falls accurately on the center of the oblique slit when the latter is turned to the horizontal position.

7. The oblique slit is turned to 45° or some other convenient angle (not horizontal nor vertical). The cylindrical lens is moved until the images from the two sides of the cell (or from the right and left sides of the schlieren lens with the center covered) coincide on the plate (except for boundaries present). If this is not possible, the plate holder is too near the oblique slit.

8. The objective lens is moved until, (a) with the object in the cell and the oblique slit vertical, the ruled lines are sharply in focus, and (b) with a symmetrical boundary gradient in the cell, the boundary (as distinguished from the base line) is symmetrical and not tipped or skewed. The previous adjustment may need to be repeated.

Finally, if there is insufficient light:

(a) open both slits to widths of 0.5 to 1.0 mm;

(b) see that the lamp is placed so that the maximum possible light falls on the schlieren lens;

(c) check the height of the horizontal slit so that its image falls squarely on the oblique slit when the latter is horizontal;

(d) see that the windows, the water of the thermostat, the solution in the cell, the light filter, and the lenses are clean.

If the base line of the image on the plate is not vertical:

(a) see that the axis of the cylindrical lens is vertical, then

(b) move the light source and slit to or from the cell until its image is in focus in the plane of the oblique slit.

If the boundary is tilted or asymmetric, but the base line is vertical and the gradient is symmetrical:

(a) see that the cell was replaced in its marked position, then if necessary

(b) move the camera objective to restore focus.

If the images of the base line for the light passing through different portions of the schlieren lens do not coincide, readjust the position of the cylindrical lens.

If the image of the boundary is complicated by diffraction effects,

giving multiple lines, try increasing the width of both slits; use a relatively short exposure time. (A properly designed double slit may be used for the oblique slit (90).)

If the line is blurred, even at small angles near vertical of the oblique slit, check the alignment of all lenses, especially that of the cylindrical lens, so that the axes coincide and the lenses are perpendicular to the axis of the system.

If the top of the boundary is cut off:

- (a) make the oblique slit more nearly vertical;
- (b) change the position of the horizontal slit sideways so that its image crosses the oblique slit with no more than enough to spare on the upper side;
- (c) use a smaller concentration gradient or wait until the gradient has spread out by diffusion.

7. Detailed Directions for Measuring Diffusion Constants Using the Standard Tiselius Electrophoresis Apparatus

a. Preparation of solution

About 13 ml. of solution are necessary—20 are more convenient—to set up a diffusion experiment in the standard Tiselius electrophoresis cell.

A concentration of 1% of protein is a good average value, the useful range of concentrations being from about 3% to 0.3%. If the protein can be protected adequately against deterioration, an economical way of planning a series of experiments is to begin with a 3% solution. After the diffusion experiment the solution may be recovered; it will be somewhat diluted and may then be repeatedly analyzed at successively lower concentrations until the useful limit of the apparatus is reached. The purpose of this procedure is to define the dependence of the diffusion constant upon concentration. Such a study is of special interest whenever the diffusion curve is skewed or flattened, in order to account for the anomaly, if possible, and is valuable in other instances. If enough material is available, the diffusion constant may be pursued to higher concentrations by adding the protein to both the solution and the buffer, keeping a convenient, say 1%, concentration difference between them. Diffusion experiments are ordinarily carried out sufficiently near the isoelectric point and at high enough salt concentrations to suppress the complicating effects of charged groups on the molecules, just as in measurements of osmotic pressure, ultracentrifugal sedimentation, and electrophoresis. Longworth (52) observed the diffusion

constant of ovalbumin to decrease by 4.4% in the pH range: 3 to 12, as measured in buffers of 0.1 ionic strength. This change is scarcely greater than the experimental variation; beyond the range of stability, however, the diffusion coefficients decreased rapidly as aggregation or unfolding occurred.

In the absence of specific complicating effects, such as interaction of the protein with some ion species present or aggregation because of insufficient solubility, it appears that any buffer within the stability range and of ionic strength 0.1—or more for especially highly charged proteins—will be suitable. Choice may then be made of a buffer which is also suitable for other studies, for example electrophoresis; or if the buffer contains no nitrogen compounds, the protein concentration of the solution may be determined conveniently by micro-kjeldahl analysis.

Preservatives are sometimes added to retard microbial deterioration of both the protein and, during storage, of the buffer. Sodium ethylmercurithiosalicylate (Merthiolate) may be used at 0.01% above about pH 4.5 at which it becomes sufficiently soluble. Toluene is commonly used to saturate both the buffer and protein solution during dialysis, although it may favor surface denaturation of the protein and is not thoroughly dependable for long periods.

If the dry protein preparation is sufficiently free from salt, as a result of dialysis, electrodialysis, or treatment with selective ion-exchange agents, it may be dissolved in a selected buffer near the isoelectric point and used at once. Ordinarily, however, a solution of a protein which is retained by a membrane of regenerated cellulose (sausage casing) is equilibrated by dialysis for a day or two against about two liters of the buffer. With care, a protein-tight knot can easily be tied in the soaked sausage casing, which is then inflated and tested for leaks under water. The bag containing the protein solution with a drop of toluene may then be closed with a similar knot under sufficient tension to prevent appreciable dilution. Drops of toluene should be removed by filtration before filling the cell.

b. Description of cell

The parts of a standard Tiselius electrophoresis cell commonly available in this country are shown in Figs. 5, 10, 11 and 12.

The cell, Fig. 5, consists of a U-tube of rectangular cross section 3 mm. \times 25 mm. (inside dimensions). The model used, with a single center section 92 mm. high, is made in three parts such that either the bottom or the two arms together up to the flared portions may

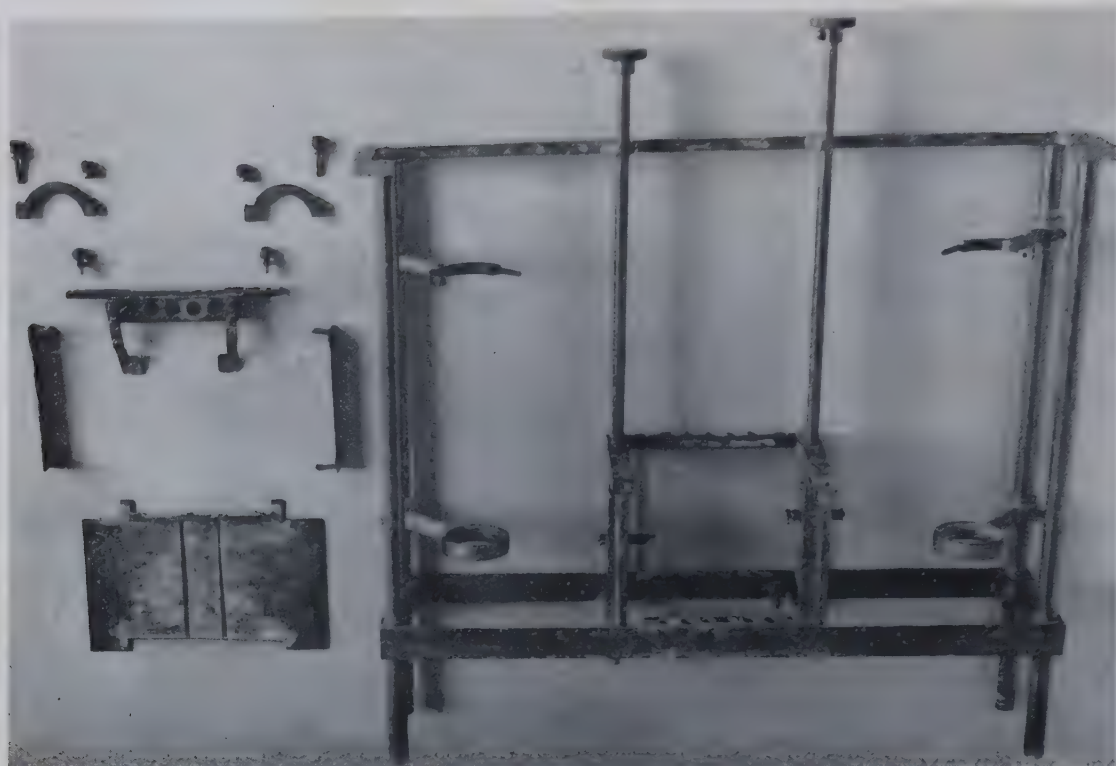


FIG. 10. Metal accessories for Tiselius cell.



FIG. 11. Glass accessories for Tiselius cell.



FIG. 12. Tiselius apparatus assembled for diffusion measurement.

be slid into or out of alignment. The 3 mm. faces of the center section are optically ground and polished.

c. Lubrication

The ground sliding surfaces of the cell are lubricated. A satisfactory traditional formula calls for melting together two parts of *yellow* petroleum jelly ("Vaseline") and one part of mineral oil. The proportions can be varied to suit special requirements. With strongly detergent solutions, or especially in the presence of fat solvents it may be desirable to use silicone stopcock grease, or, since this is quite stiff, Dow-Corning fluid no. 200 of viscosity either 7600 or 1000 (centistokes at 25°). These silicones are satisfactory as lubricants but are troublesome to clean from the cell. In any case the lubricant should be spread thinly and uniformly over the sliding surfaces in such a way that no air spaces are left to work air

bubbles into the channels when the sections are moved back and forth, but also in such a way that excess lubricant is not squeezed out into the channels.

d. Cleaning

If the cell is clean and dry before assembly, it need not be rinsed with the solution to be analyzed. After the experiment it may be rinsed with buffer followed by water and kept in the assembled condition either filled with water or drained and dried. In the first case it must be rinsed with the next solution to be analyzed; in either case the walls of the channel soon become greasy enough to hold air bubbles obstinately and the sliding surfaces acquire a tendency to stick. It is therefore, good practice to clean the cell between analyses.

e. Assembly and filling of the cell

The sliding faces of the cell are lubricated as described and gently worked together to remove air spaces between the plates.

The vertical brass strips which are used in moving the center section are put in place and the cell is set in the holder. A systematic assembly procedure is recommended with reference marks assuring that the cell is always put together with all parts in the same relative position in the holder.

A mask of sheet brass, not standard equipment (see Fig. 10), is placed in position and the top clamp of the holder is put in place. Masking may also be done with opaque photographic masking tape on the front (nearest the camera) window of the constant temperature bath, in order to cut off light not passing through the cell when it is in position.

The channels of the cell are aligned. The filtered solution is slowly poured down the wall of the right channel (the mask and open side of the holder being toward the experimenter). In particular instances it may be desirable to bring the cell and solution to the temperature of the bath before filling; or it may be helpful to subject the solution and buffer (in suction flasks) to reduced pressure in order to minimize bubble formation later, especially when a refrigerated solution is analyzed above room temperature. Bubbles at the bottom joint and in the bottom section are dislodged, if possible, with a clean wire bent at the end, taking care not to scratch or get lubricant on the narrow faces of the center section.

The cell is then tipped if necessary, so that the solution fills the bottom section and the left side of the center section. The bottom

section is pushed to the left (with the right rack-and-pinion-operated pushing arm, the left pushing arm being set at the bottom of the brass strip on the center section) while the cell is in this position. The sealed off joints are inspected to assure the absence of bubbles.

Superfluous solution is removed from the right side of the cell, which is then rinsed three or four times with the buffer solution and finally filled with the buffer. An 8-inch, blunt, 17 gauge, rustless, hollow needle used with a 20 ml. standard syringe is of great assistance in rinsing, care being taken not to get lubricant on the narrow faces of the center section. Bubbles are dislodged with a clean wire as before. The center section is then pushed to the right as far as it will go, placing the left pushing arm in the center of the brass strip. and the bottom section is returned to the center position against its stops with the same left pushing arm.

The left side of the upper section is next rinsed three or four times with buffer and both sides of the upper section are filled with buffer.

f. Attachment of the electrode vessels

No electrodes are used in diffusion experiments, but the electrode vessels of the Tiselius apparatus must nevertheless be attached unless some modification is made in the apparatus permitting it to be closed off from the bath and permitting movement of the boundary into position ("compensation").

Rubber or neoprene sleeves may be used to join the electrode vessels to the cell. Penrose rubber drain tubing, $\frac{7}{8}$ " in diameter, obtained from surgical supply houses in yard lengths may be used. Apparatus with ground glass joints is more satisfactory when available.

The clamps holding the electrode vessels are adjusted to give the least possible clearance (without contact) between the electrode vessels and the cell when they are in alignment. The ground joints of the vessels are lubricated with the Vaseline mixture. Both sleeves are rinsed in the solvent, slipped on the appropriate connecting tubes of the electrode vessels and held in place with several turns of a good quality rubber band (e.g. pure crepe, size 62). A second rubber band folded twice or three times is slipped over the sleeve to the glass, where it is easier to get hold of again. The sleeve is then entirely and neatly pushed up onto the connecting tube of the electrode vessel.

The electrode vessel is filled with buffer up to the point of attachment of the side arm; it is then placed and clamped (not perfectly

rigidly) in position. Too much play in the holder may be corrected by padding the vessel with rubber sleeving. The free end of the connecting sleeve is then slipped down over the top of the cell, smoothed out to remove wrinkles and bubbles, and held in place on the cell by means of the second rubber band. The second electrode vessel is treated in the same way.

The electrode vessel with the two ground joints is conventionally attached to the left side of the cell and the one with the single ground joint to the right side. Buffer is then added to fill the electrode vessels up to the ground joints. The right-hand-most ground joint is closed by a #24 standard taper glass stopper and the left-hand-most by a #27 standard taper glass stopper in place of the electrode assembly. The right-hand opening for the electrode is covered with a loose-fitting composition cap or dust cover allowing access to atmospheric pressure.

The second ground joint, on the left-hand electrode vessel, over the connecting tube to the cell, is used for attachment to the compensating device used to move the liquid in the cell at a rate slow enough to avoid much mixing.

The simple funnel and three-way (T) stopcock arrangement shown is used to join the electrode vessel to the syringe of this compensator as follows. The stopcock is closed. The funnel is filled with buffer. The stopcock is fully opened long enough to rinse air bubbles from the filling tube to the funnel and turned to connect the long rubber tube to the ground joint. The free end of the rubber tube is rolled back on itself (over a rod) in order to help get it over the tip of the syringe. The tube and joint are freed of buffer. The left-hand electrode vessel should be completely filled with buffer. The ground joints of the funnel attachment and the electrode vessel are then put together with gentle suction through the rubber tube only just sufficient to fill the stopcock, which is then closed when the pieces are together. The joint is fixed in place with a rubber band. The cell assembly is shown in Fig. 12.

The next step is to put the apparatus in place in the constant temperature bath. The stopcock is then turned temporarily to connect the funnel with the rubber tube until the latter is completely filled with buffer. The tube is slipped over the end of the syringe of the "compensating" device described in the next paragraph. The stopcock is opened to join all three arms while the apparatus is coming to bath temperature—about 45 minutes; it is turned to join the funnel and syringe, isolating the cell, with care to avoid putting stress on the apparatus, while the center section is slid into

place, forming the boundaries or whenever the syringe of the compensator is to be tested for freedom of motion; or it is carefully turned to join the cell with the syringe for compensating. The stopcock can be turned to join the compensator with the cell or to isolate the cell in any of these manipulations without necessarily connecting the cell with the filling tube; when the cell is properly isolated, the stopcock is turned so that the inlet to the cell is sealed off with the maximum distance of lubricated ground surface.

g. "Compensation"

In order to move the boundaries into observable positions after they are formed, a synchronous motor geared down to one-fifth of a revolution per minute actuates a standard 50 ml. glass syringe through a shaft threaded with 50 threads per inch. Under these conditions the syringe pumps buffer into or out of the cell at a rate of nearly one cubic centimeter in 17 minutes. The mechanism is standard equipment and works well if carefully lubricated. The plunger of the syringe should also be lubricated. The syringe itself should be clamped as lightly as possible. With this arrangement, both boundaries are moved into view in eight to ten minutes, and to the center of the center section in 40 minutes to an hour after the "compensation" is started.

A pair of limit switches to turn off the compensator motor after a predetermined amount of travel is a useful addition to the commercially available apparatus. These may be microswitches actuated by a nut on the bottom of the threaded shaft.

h. Beginning the experiment

When the cell has been in the constant temperature bath for 45 minutes or an hour the boundaries are ready to be formed. The piston of the syringe compensator is first tested for freedom of action and adjusted if necessary, the stopcock is turned to isolate the cell from the compensating syringe and funnel; and the center section is slowly slid into alignment with the other sections. The time is noted and the timer started.

A synchronous clock which must be reset to start in case of a power failure is recommended, in conjunction with a synchronous timer reading to seconds. If the experimenter wishes, he may start the timer from the ten second, or some other reading, to allow for the interval between manipulating the rack-and-pinion to slide the center section into place and starting the timer. This is unnecessary, however, because the experimentally observed times are

modified to allow for the disturbance inherent in forming the boundary by reckoning from a computed "zero time," as described later.

From the time the boundaries are formed, the stopcock must not be turned, even momentarily, so that the cell is joined to the funnel. The stopcock is then carefully turned to join the cell to the syringe. The compensator is started in the proper direction to move the boundaries into view (pushing the plunger into the syringe). In 40 to 60 minutes, when the boundaries have reached the center of the plate as determined with the help of a ground glass screen, the compensator is stopped and the cell isolated by turning the stopcock.

i. Making the records

The initial photograph should be taken soon after stopping the compensation. Eastman spectroscopic plates, type III-G, with antihalation backing, may be used with a mercury vapor lamp and filter, for example, the Corning combination consisting of filters no. 4303 (3.95 mm.), 3486 (3.40 mm.), and 5120 (5.07 mm.), optically ground and polished; or Wratten filter no. 77A, isolating the green line of 0.546 micron wave length.

Faster and slower spectroscopic plates with a great variety of sensitizations are available and may be used, or for general purpose work, especially where light sources of various wave-lengths will be used, any reasonably fast panchromatic plate, for example, Kodakline CTC Pan or Wratten M. (Fast orthochromatic plates have been suggested for use without filters (89).)

A series of test exposures is necessary on first experience with a given light source and optical arrangement; for the cylindrical lens or scale methods, begin with a one-second exposure and make each trial exposure twice the preceding, up to 10 minutes. If an exposure of a minute or more seems necessary, with a clear protein solution, the optical system should be checked very carefully for correct adjustment, as described. Probably several exposures will seem equally satisfactory, the choice resting upon the contrast and clarity of the record. Longer exposures give more confusion from interference patterns. The manufacturer's recommendation should be adhered to in processing the plates.

With the cylindrical lens methods, the resolution is favored by using the diagonal diaphragm as nearly horizontal as practicable. However, it should not be so nearly horizontal that the top of the figure is open or cut off. The pattern will in general be easier to measure if it is not sharp on top.

Reference to the formula relating the diffusion constant to the height of the curve and the time shows that the height varies inversely as the square root of the time.

This means, for example, that if the first picture can be taken 2500 seconds after the boundary is formed and a measurable difference in height takes place by about 3000 seconds, the height will decrease successively by the same amount at the successive times shown in Table II, which may serve as a guide for making exposures.

TABLE II
THE DECREASE WITH TIME OF THE HEIGHT OF AN IDEAL
CONCENTRATION GRADIENT

Time (Seconds)	Time (Days, Hours, Minutes)	$1/\sqrt{t}$ (= $K \times \text{Height}$)
0	0 min.	
2,500	42 min.	0.020
3,086	51 min.	0.018
3,906	1 hr. 5 min.	0.016
5,102	1 hr. 25 min.	0.014
6,944	1 hr. 56 min.	0.012
10,000	2 hr. 47 min.	0.010
15,625	4 hr. 20 min.	0.008
27,778	7 hr. 43 min.	0.006
62,500	17 hr. 22 min.	0.004
250,000	2 days 21 hr. 27 min.	0.002

Note that as the time is increased by the factor X^2 , the height is decreased by the factor $1/X$, for a given slit angle.

This means that photographs should be taken at relatively frequent intervals at the beginning of an experiment, and decreasingly often thereafter, the practical limit of the experiment being when the gradient extends to the limit of observation of the plate. This condition is not always easy to estimate; a practical criterion is the consistency of values found for the area under the curve.

Table II suggests a schedule of photographs which should give good results for proteins of molecular weights of the order of 30,000 to 100,000. It is not necessary to follow it exactly. Computation is slightly simplified by taking photographs at times having as high powers of 10 as possible as exact factors.

8. Computation and Interpretation of Diffusion Data from an Experiment in Free Diffusion

Summary

1. Determination of the photographic enlargement factor.
2. Preparation of the data or records for study: plotting line displacement data against position in the cell—for the scale method;

making enlargements of known scale from mechanical scanning or cylindrical lens schlieren records; introducing coordinates.

3. Determination of the diffusion constant $D_{0,h}$, from the maximum ordinate and area between the experimental curves and the base lines.

a. the measurement of the maximum ordinate and area and their ratio.

b. determination of the diffusion constant $D_{0,h}$, from A and H; graphical representation to show consistency of data and the use of the method of least squares to determine the best straight line to represent the data.

4. Determination of the diffusion constant $D_{2,0}$, by the method of moments.

a. tabulation of the data.

b. determination of the axis of the curve and of the second moment about this axis.

c. determination of the enlargement factor.

d. reference of the data to "zero time." The determination of $D_{2,0}$.

5. The diffusion of mixtures. "Shape analysis" of the diffusion curve.

6. Reference of diffusion constants to standard conditions.

7. Estimation of molecular weight from diffusion constants.

a. by the Stokes-Einstein treatment.

b. with the use of the frictional resistance coefficient independently determined; with the assistance of sedimentation data.

a. Determination of the photographic enlargement factor

The photographic enlargement factor $1/F$ is the quotient of an image length on the photographic plate divided by the corresponding object length in the diffusion cell. This factor is determined experimentally for the direction of the diffusion process. A suitable object, which may be a 2 mm. strip cut from a fogged and processed photographic plate on which a recognizable pattern of ruled scratches or cut out spaces has been prepared, is mounted in the cell. In this instance the gelatin may be hardened or varnished so that it will not be displaced when wet. The object is fixed in the cell so that its ruled surface is at a distance $0.423a$ ($= a - a/\sqrt{3}$) from the inside of the cell window nearest the photographic plate (66, p. 195). This is for the schlieren methods; the enlargement from the cell is found from the geometry of the apparatus for the scale (48) method. In this formula a is the thickness of the cell in the direction

of the optical axis of the system. The glass strip may be held in place against smooth blocks of bakelite of appropriate dimensions by round rubber discs cut from a stopper. The cell, filled with water or buffer solution, is then placed in its exact position in the constant temperature bath and one or more photographs (with various exposure times) taken of the scale. If the mechanical scanning method is used, the diaphragm should be out of the way and not used in this calibration. If the cylindrical lens method is used, the slit should be vertical (angle: 0°). Corresponding distances on the object strip and on its photograph, may be conveniently measured and compared with a traveling microscope or micrometer slide comparator.



FIG. 13. Record of diffusion gradient: 2% bovine serum albumin in pH 4.7, ionic strength 0.1, sodium acetate buffer at 30° , 100,100 seconds after forming the boundary.

b. Preparation of the records for study: schlieren scanning or cylindrical lens methods

Schlieren photographs obtained by either mechanical scanning or by cylindrical lens methods will have the general form of the normal curve of error and present graphs of the refractive index gradient (and therefore the concentration gradient) as a function of position in the cell. Copies of the records are made with a good enlarging camera at a linear enlargement of three to ten times. An ordinary enlargement is sufficient to find the diffusion constant $D_{0,h}$, as described in section III8c, below. It is convenient, however, to make the projection print from the plate with its emulsion side in contact with a truly rectangular negative transparency of a sheet of coordinate paper as in Fig. 13. A convenient scale is 30 divisions per centimeter on the plate. Such an arrangement serves to check the precision of the enlarger and gives a convenient measure of the degree of enlargement from the plate to the paper (this degree of enlargement need not be determined explicitly); the dimensions of the units on the coordinate transparency being precisely meas-

ured, diffusion constants may be computed in terms of the coordinate units at any degree of secondary enlargement and referred directly to the true physical dimensions by means of the same constant factor. The primary purpose of the scale, however, is to allow tabulation of the ordinates of the curve as a function of vertical position in the cell; this is necessary for computing weight-average diffusion coefficients, $D_{2,0}$, and for "shape analysis," it being desired to compare the shape of the experimental curve with that of an ideally diffusing, homogeneous substance. It is also possible to work from carefully made tracings on graph paper of an enlarged image; in this case the degree of enlargement may be computed from the measured lengths of the whole record and its tracing.

c. Determination of the diffusion constant $D_{0,h}$, from the maximum ordinate and area between the experimental curves and the base lines

(1) The measurement of the maximum ordinate and area and their ratio.

(a) Determination of the base line

The enlarged copies of the diffusion records from either the mechanical scanning or cylindrical lens method are next examined to determine the position of the base line. A preliminary "blank" study in the absence of a refractive gradient in the cell shows whether the base line may safely be considered to be straight and "horizontal," or if not, what deviation is present.

If the base line is satisfactorily ideal, it may be drawn in, to a sufficient approximation, tangent to the undeviated portions of each enlarged record with a straight-edge and sharp, hard lead pencil. If it is not ideal, one possible method of treatment is to cut a corresponding template from an enlargement to the same scale as the enlargement of the other records with some registration mark clearly indicated, possibly one end of the record—and to use this template to draw in the base line. Analysis of the exact form of the curve is sometimes made difficult by diffraction effects. These are analyzed, for the mechanical scanning method, by Longworth (53).

In the case of records from the cylindrical lens method using a diagonal slit, one is faced with a choice of using the top of the line, which may be quite broad, the bottom of the line, or a midpoint—as suggested, for example, by Cooper (cited by Neurath and Putnam (64)). However, if the peak is not too sharp either the top or the bottom of the record line will yield a result satisfactory to two

significant figures, when following the treatment indicated. The base line is drawn in to match the top or bottom of the record line at its extremities, as appropriate.

(b) Measurement of the maximum height, H

The maximum height of each record, from the maximum of the curve perpendicularly to the base line, is next measured. A centimeter scale may be used; it is convenient, however, if the enlargement is made with coordinates to record the height in terms of coordinate units, which may be read directly if the curve is square with the coordinates.

(c) Measurement of the area, A

The area enclosed between the base line and the record line of each figure is determined. Three methods are suggested.

By weighing

For a quick approximation the area may be carefully cut out and weighed, and the weight compared with that of a portion of known area having a standard geometrical shape. A triangular metal template of known dimensions including as much of the figure as possible may be used with a razor blade.

With a polar planimeter

The area may be measured with a polar planimeter. For example: the record is mounted with drafting tape on a smooth, uniform surface. The planimeter is set up so that the joint is approximately rectangular when the tracing point is in the center of the figure. The wheel and the supporting pin should not strike any irregularity during their movement. The tracing point is set on some recognizable reference point on the perimeter of the area and a reading taken. The perimeter is then traced as smoothly as possible back to the starting point with care to keep the wheel against the surface at all times. A second reading is then taken and the two subtracted to get the area in planimeter units. (If the zero point is passed once during the tracing, the digit *one* must be supplied as the first significant figure of the minuend; the minuend will be the second reading if the reading increases during the tracing or the first reading if the reading decreases during the tracing.)

The area may be measured six or eight times, tracing alternately clockwise and counterclockwise. The different values agree at least to 2 to 5%. A triangle of known dimensions may then be drawn covering the same general area and measured to calibrate the pla-

nimeter under the working conditions. The results should be converted to the units (squared) used for measuring the height; if the coordinate method is used, the coordinate unit square is a convenient unit.

By counting squares

The third method, depending upon the use of coordinates, is to count squares. This is probably not worthwhile of itself, but is recommended when shape analysis is carried out, as discussed in Section 5; and indeed, the data are collected incidentally in the process.

(d) The ratio A^2/H^2

The ratio of the area to the maximum height is then computed and recorded, together with the square of this ratio. This square changes with time in direct proportion to the diffusion coefficient. The ratio A^2/H^2 is independent of certain apparatus constants. The factors which change the height of the curve—the velocity of the photographic plate and diaphragm for the mechanical scanning method, the angle of the diaphragm for the cylindrical lens method—change the area only in direct proportion, so that the ratio is unaffected by these apparatus constants. If, for any reason, one wishes to compare the areas, for example at different slit angles, for the cylindrical lens method, the areas, like the heights, are directly proportional to the tangent of the angle of the slit from the vertical position (perpendicular to the illuminating slit).

(2) Determination of the diffusion constant $D_{0,h}$ from A and H

The diffusion constant is related to the area and maximum height as just determined (Wiener's method) by the formula

$$D_{0,h} = \frac{\omega^2 A^2}{4\pi t H^2} \quad (V)$$

in which the subscripts 0 and h together denote the computation from the area and height, respectively A and H, t is the elapsed time, and ω (centimeters in the cell per enlargement unit) is the linear enlargement factor relating units of measurement on the enlargement to the units of measurement in the cell (centimeters).

(a) Units

If A and H are measured in graph units, ω is the number of centimeters in the cell corresponding to the linear graph unit, and t is in seconds, D will have the units: centimeters squared per second.

(b) The evaluation of the enlargement factor ω

Case 1. If the values of A^2/H^2 have been found in terms of centimeters on the enlarged curve: let a_3 centimeters on the enlargement correspond to a_2 centimeters on the photographic plate and to a_1 centimeters in the cell as measured experimentally. One centimeter on the plate will correspond to a_1/a_2 centimeters in the cell, or F as stated in section III8a. The enlargement of the print from the plate may be given as a_2/a_3 , which may be called R , the number of centimeters on the plate corresponding to one on the print. One centimeter of the enlargement corresponds to FR centimeters in the cell and $\omega = FR = a_1/a_3$.

Case 2. If the values of A^2/H^2 have been found in terms of arbitrary coordinate units: let a_2 centimeters on the photographic plate correspond with a_1 centimeters in the cell as above. Let n rulings per centimeter be the spacing of lines on the coordinate grid from which, in contact with the plate, the enlargement was made. It is supposed that the unit chosen was the coordinate unit as projected. One centimeter on the plate will correspond to a_1/a_2 , or F , centimeters in the cell, as before, and this in turn to n coordinate units, no matter what the degree of enlargement is. One unit of the enlargement corresponds to F/n centimeters in the cell and $\omega = F/n = a_1/na_2$.

(c) Decrease with time: allowance for initial boundary disturbance

If the diffusion constants computed by the formula just given are plotted against time it will generally be found that there is a definite trend of the values with time. This is attributed to disturbances in forming the boundary, giving in effect a "zero time" earlier than the actual starting time of the experiment. This zero time and the corresponding diffusion constant $D_{0,h}$, may generally be computed in a straightforward way, on the assumption that

$$D_{0,h} = \frac{\omega^2 A^2}{4\pi(t+t_0)H^2} \quad (\text{VI})$$

as follows.

For the purposes of computation, equation (VI) is put into the form

$$\frac{\omega^2 A^2}{4\pi H^2} = D_{0,h}t - D_{0,h}t_0 \quad (\text{VII})$$

which is that of a straight line,

$$y = mx - b \quad (\text{VIII})$$

where $\omega^2 A^2 / 4\pi H^2$ corresponds to y ; t to x ; and $D_{0,h}$ is the slope m ; $D_{0,h}t_0$ is the y -intercept; and $-t_0$, the x -intercept, gives the zero time.

Graphical treatment

The first of three ways of treating the data is graphical. The quantity $\omega^2 A^2 / 4\pi H^2$ is plotted as a function of t , the measured elapsed time. A straight line is fitted by eye to the plotted points. The diffusion constant $D_{0,h}$ is given by the slope of this line, that is, the change in $\omega^2 A^2 / 4\pi H^2$ corresponding to a change in t of one second.

Algebraic simple check

Secondly, an algebraic check upon the graphical method consists of selecting two pairs of data (t , $\omega^2 A^2 / 4\pi H^2$) which appear to be representative, that is, for example, which lie on the estimated straight line. The difference in $\omega^2 A^2 / 4\pi H^2$ divided by the difference in t gives the diffusion coefficient $D_{0,h}$.

Method of least squares

Finally the best straight line may be found by the method of least squares as follows (a computing machine, such as the Friden full automatic tabulating calculator, Model ST, is an invaluable time saver both here and in the procedures described in the following section): A table is made of all reliable data pairs: t and $\omega^2 A^2 / 4\pi H^2$. The number of data pairs considered, n , is recorded, and the following sums are found (" Σ " denotes "the sum of all quantities of the following form"):

$\Sigma(t) = T$: the sum of all n values of t ;

$\Sigma(\omega^2 A^2 / 4\pi H^2) = K$: the sum of all n values of $\omega^2 A^2 / 4\pi H^2$;

$\Sigma(t^2) = L$: the sum of all n values of the squares of t ;

$\Sigma(t \cdot [\omega^2 A^2 / 4\pi H^2]) = M$: the sum of all n products of the individual data pairs.

The quantities D , N_a , and N_b are now found:

$$D = \begin{vmatrix} n & T \\ T & L \end{vmatrix} = n \cdot L - T^2 = n\Sigma(t^2) - (\Sigma(t))^2 \quad (\text{IX})$$

$$\begin{aligned} N_a &= \begin{vmatrix} K & T \\ M & L \end{vmatrix} = K \cdot L - T \cdot M \\ &= \Sigma \left(\frac{\omega^2 A^2}{4\pi H^2} \right) \cdot \Sigma(t^2) - \Sigma \left(t \cdot \frac{\omega^2 A^2}{4\pi H^2} \right) \cdot \Sigma(t). \end{aligned} \quad (\text{X})$$

$$N_b = \begin{vmatrix} n & K \\ T & M \end{vmatrix} = n \cdot M - T \cdot K = n\Sigma \left(t \cdot \frac{\omega^2 A^2}{4\pi H^2} \right) - \Sigma \left(\frac{\omega^2 A^2}{4\pi H^2} \right) \cdot \Sigma(t). \quad (\text{XI})$$

$D_{0,h}$, the slope, is N_b/D . The intercept on the time axis is N_a/N_b . These computed values may be checked against the graphical representation to avoid gross errors.

d. Determination of the weight-average diffusion constant $D_{2,0}$, by the method of moments (48)

Condensed summary*

(1). Reduce the data to tabular form giving the refractive index gradient (S) in arbitrary units as a function of distance (s) at uniform intervals in the line of diffusion.

(2). Compute the following sums, including all data different from zero. $N = \Sigma(S)$, $\Sigma(sS)$, $\Sigma(s^2S)$; compute $\beta = \Sigma(sS)/\Sigma(S)$ and β^2 , and $\sigma^2/\omega^2 = \Sigma(s^2S)/\Sigma(S) - \beta^2$.

(3). Determine ω , the distance in the cell corresponding to the unit value selected for s ; compute ω^2 and σ^2 .

(4). Take the zero time correction from section 3b, or better, compute it in the same way using $\sigma^2/2$ in place of $\omega^2 A^2/4\pi H^2$ as a function of time.

(5). Compute

$$D_{2,0} = \frac{d}{dt} \left(\frac{\sigma^2}{2} \right) = \frac{\sigma^2}{2t}$$

where t in the second instance is taken from the zero time determined.

(1) Tabulation of the data

Figure 13 shows a selected record of a diffusion experiment in which a boundary formed between a solution of bovine serum albumin in pH 4.7, 0.1 ionic strength buffer (containing 0.1 N -sodium acetate and 0.1 N acetic acid) was allowed to diffuse 100,100 seconds. Records were made by the Philpot-Svensson cylindrical lens method and the plate was printed by projection in contact with a negative of coordinate paper (as described in section III8b) having the lines 0.0309 cm. apart. If there is any variation in spacing, one should select the value measured as near the position of the actual record as possible and in the direction of the diffusion process, *i.e.*, in the direction of the base line. The curve is almost square with the coordinate rulings. The heavy ruling next below the curve is selected as the tentative zero of ordinates, from which the heights along the curve are measured. The marked ruling near the position of the maximum height is selected as the tentative zero

of abscissas; the exact choice is unimportant. A data sheet, Table III, is now begun by noting values of all abscissas at selected unit intervals covering the range in which the experimental curve differs from the base line; ordinarily the unit chosen will be one division of the coordinate rulings, but if the curve is very broad it is permissible to choose two or even more divisions as the unit, so that the total number of ordinates different from the base line values will be of the order of one-hundred to one-hundred fifty. A consequence of this selection is to make negligible Sheppard's correction mentioned later. These abscissas at unit intervals are the *s*-values.

The ordinate corresponding to each abscissa is recorded. The ordinates may be distances in the coordinate units from the tentative base line to the place at the top of the curve at which the rulings are judged to become just visible. The bottom of the curve may be treated in the same way. In the illustration given, Table III, the average of the top and bottom values was chosen. In any case, the values of the ordinates as just measured are corrected with reference to the position of the true base line with respect to the refer-

TABLE III

SAMPLE DATA AND COMPUTATION OF THE WEIGHT AVERAGE
DIFFUSION CONSTANT, $D_{2,0}$, BY THE METHOD OF MOMENTS

(A) *Data for the diffusion constant of bovine serum albumin*
(Armour)

Solvent: pH 4.7, ionic strength 0.1 buffer containing 0.1 *N* sodium acetate and 0.1 *N* acetic acid.

Protein concentration: 2%.

Notes: The bath temperature was 30.0°, measured by a short scale, jacketed thermometer. Variations in temperature were observed with a Philadelphia differential thermometer, the readings of which are recorded as "° D."

The exposure record gives the plate holder, the position on the plate, and the arm of the cell photographed.

The boundaries were formed Mar 4 at 12:51. The timer was started at 12:52. The boundaries were compensated into position by 13:26 and the cell isolated.

A record of the experiment was kept in the following form.

Observation No.	Temperature	Slit Angle	Exposure	Date	Time	Timer (sec).
1	2.418° D	10°	2B-10-R	3/4	14:33	6,000
2	to 2.422	10°	2B-9-L			6,500
13	2.418° D	30°	3A-8-R	3/5	11:23	81,000
14	2.418° D	30°	3A-7-L		16:41.5	100,000
19		45°	3A-2-R	3/10	10:50	511,000

A print from the fourteenth exposure record is shown in Fig. 13 and analyzed in the following parts of this table.

TABLE III—Continued

(B) Tabular form for measured and computed quantities

s^2	s	[S _l]	[S _u]	[S _m]	S	(s- β)	$\omega(s-\beta)$	σS
							σ	ωN
4624	-68	5.3	3.1	4.2	0.0	-71.6		0.000
4489	-67	5.4	3.1	4.3	0.1	-70.6	-3.688	0.002
4356	-66	5.5	3.1	4.3	0.1	-69.6	-3.636	0.002
4225	-65	5.4	3.2	4.3	0.1	-68.6	-3.584	0.002
4096	-64	5.4	3.1	4.3	0.1	-67.6	-3.531	0.002
3969	-63	5.4	3.1	4.3	0.1	-66.6	-3.479	0.002
3844	-62	5.6	3.0	4.3	0.1	-65.6	-3.427	0.002
3721	-61	5.6	3.1	4.4	0.2	-64.6	-3.375	0.003
3600	-60	5.2	3.3	4.3	0.1	-63.6	-3.322	0.002
3481	-59	5.6	3.2	4.4	0.2	-62.6	-3.270	0.003
3364	-58	5.5	3.2	4.4	0.2	-61.6	-3.218	0.003
49	-7	27.6	24.2	25.9	21.7	-10.6	-0.554	0.349
36	-6	28.0	25.0	26.5	22.1	-9.6	-0.502	0.356
25	-5	28.3	25.5	26.9	22.7	-8.6	-0.449	0.365
16	-4	28.8	26.0	27.4	23.2	-7.6	-0.397	0.373
9	-3	29.3	26.0	27.7	23.5	-6.6	-0.345	0.378
4	-2	29.3	26.2	27.8	23.6	-5.6	-0.293	0.380
1	-1	29.9	26.5	28.2	24.0	-4.6	-0.240	0.386
0	0	30.5	27.4	29.0	24.8	-3.6	-0.188	0.399
1	1	30.6	27.4	29.0	24.8	-2.6	-0.136	0.399
4	2	30.7	27.5	29.1	24.9	-1.6	-0.084	0.401
9	3	30.5	27.4	29.0	24.8	-0.6	-0.031	0.399
16	4	30.5	27.4	29.0	24.8	0.4	0.021	0.399
25	5	30.3	27.5	28.9	24.7	1.4	0.073	0.397
36	6	30.4	27.3	28.9	24.7	2.4	0.125	0.397
49	7	30.3	27.0	28.7	24.5	3.4	0.178	0.394
64	8	30.0	26.6	28.3	24.1	4.4	0.230	0.388

Notes: This table shows in part the data derived from Fig. 13. In it, s is the value of the arbitrary abscissa assigned to a coordinate ruling, choosing zero near the position of the maximum of the curve. The corresponding ordinates measured from an arbitrary base line to the lower and upper edges, and (by averaging) the middle of the record line are given as [S_l], [S_u], and [S_m]. The minimum consistent value of [S_m] is 4.2 at either end of the record; 4.2 is therefore subtracted from each [S_m] value to give S, the ordinate used in the further computation. The last two columns give the normal coordinates, in terms of which the area under the curve and its standard deviation are both unity, permitting an immediate comparison (parts D, E, this table) with the normal diffusion curve.

ence base line used in measurement. In the example, it is seen that the curve at the extreme left and right sides has the value 4.2. In many cases it can be shown that at the slit angle and other optical arrangements used, the base line is for practical purposes a straight line in the region covered by the boundary. Therefore, 4.2 is subtracted from all ordinates. If the curve is not quite square with the coordinates, different values must be subtracted from the left-most ordinate and from the right-most, and the corrections for the intermediate ordinates determined by linear interpolation. The corrected ordinates found in this way are the S-values.

TABLE III—*Continued*

(C) *Summary of the computation of $D_{2,0}$ from the data tabulated in part B, this table*

$\Sigma(S) = N = 1,189.8 = A$, the area under the curve in the coordinate units.

$$N^2 = A^2 = 1,415,624.$$

$$\Sigma(sS) = 4,242.5.$$

$$\frac{\Sigma(sS)}{N} = \beta = 3.5657.$$

$$\beta^2 = 12.714.$$

$$\Sigma(s^2S) = 451,102.9.$$

$$\frac{\Sigma(s^2S)}{N} = 379.142.$$

$$\frac{\Sigma(s^2S)}{N} - \beta^2 = 366.428 = \frac{\sigma^2}{\omega^2}.$$

One cm. on the photographic record corresponds to 0.68162 cm. in the cell = F.
The coordinate grid used has 32.36 lines per cm. = n.

$\omega = F/n = 0.02106$ cm. in cell per coordinate unit.

$$\omega^2 = 4.436 \cdot 10^{-4}$$

$$\sigma^2 = 16.238 \cdot 10^{-7} \text{ cm.}^2$$

$$D_{2,0} = \frac{\sigma^2}{2t} = 8.1 \cdot 10^{-7} \text{ cm.}^2 \text{ sec.}^{-1}$$

(D) *Test of homogeneity*

(1) Ideally diffusing substances give diffusion curves having the form of the normal probability curve. For such substances, diffusion constants computed by methods giving different sorts of average values are found to be the same. As a test of homogeneity and the ideal character of the diffusion process, it is convenient to compare $D_{2,0}$ with $D_{0,h}$, the ratio of which may be found from the data tabulated, in part, in part B, without evaluating the enlargement factor or the elapsed time, as follows.

The maximum ordinate is $H = 24.9$ units. $H^2 = 620.01$.

$$\frac{D_{2,0}}{D_{0,h}} = \frac{\sigma^2}{\omega^2} \cdot \frac{2\pi H^2}{A^2} = 1.008.$$

The excess of this ratio above unity is a measure of polydispersity.

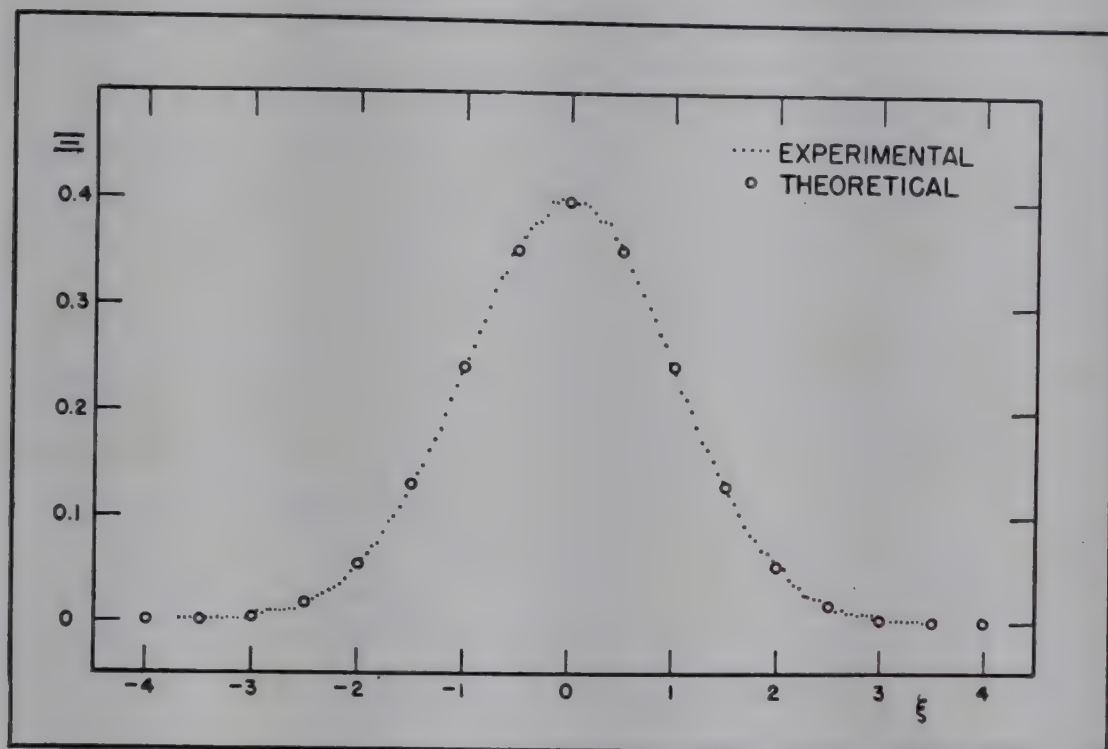
(2) A more detailed test of ideal behavior is the comparison of the experimental curve in normalized coordinates (part B) with the normal probability curve, as illustrated by the graph, part E, this table.

(2) *Determination of the axis of the curve and of the second moment about this axis*

The products sS and s^2S may now be tabulated for each recorded S -value as a preliminary to finding the three sums (taking into ac-

TABLE III—Continued

(E) Graph of the concentration gradient or refractive index gradient as a function of position in the cell, in normalized coordinates, for comparison with the normal probability curve, represented by the larger circles



(F) Reference of $D_{2,0}$ to the standard conditions: 20° in pure water

In making the corrections, it is helpful to recall that the diffusion constant will be less at 20° than at 30° through the operation of each of two factors, but will tend to be increased by the removal of salt from the medium because of the slight attendant decrease in viscosity.

The correction factors for the case at hand are: (1) for the effect of temperature on molecular motion, $\frac{293.18}{303.18}$, (2) for the increase in the viscosity of the medium with the decrease in temperature, 0.7935, the ratio of water at 30° relative to that at 20° (reference 90, Appendix I), (3) for the decrease in viscosity attendant upon the removal of salt and acid, 1.048, the viscosity relative to water at the same temperature (20°) of a solution containing both 0.1 *N* sodium acetate and 0.1 *N* acetic acid, as derived from reference 90, Appendix III, taking the increments in relative viscosity to be additive.

The combined correction factor is 0.80416.

$D_{2,0}$ at 20° in pure water is then computed to be $6.5 \cdot 10^{-7}$ cm.² sec.⁻¹.

The diffusion constant of bovine serum albumin is reported (89) to be $6.0 \cdot 10^{-7}$. The discrepancy is eliminated in this instance by evaluating the zero time as described.

count algebraic signs) of all the recorded S-values different from zero of the corresponding sS-values, and of the corresponding s^2S -values. If an automatic tabulating computing machine is available these individual products need not be written explicitly, but the sums may be found directly and checked. This should be done systematically, and partial sums noted at regular intervals, say every 10 numbers, to help in finding errors in computing. A table of the squares of the integers will be found useful.

The quantity β^2 is now computed. Beta (β) is the distance in graph units by which the tentative axis of ordinates failed of passing through the centroid, or "center of gravity" of the figure; β is defined by the formula $\beta = \Sigma(sS)/\Sigma(S)$.

The ratio $\Sigma(s^2S)/\Sigma(s)$ is subject to an error, generally negligible, due to the use of finite intervals for s in the summation (97). The appropriate correction, Sheppard's correction, requires subtraction of $h^2/12$, where h is the width of the unit interval chosen for s ; that is, h equals unity in our instance because the computation has been done in terms of the coordinate unit.

The value σ^2/ω^2 which may be described as the "variance," or "square of the standard deviation" of the diffusion curve in terms of the coordinate unit, is the second moment of the curve about the true axis through the centroid referred to a figure of unit area. It is given by the formula:

$$\frac{\sigma^2}{\omega^2} = \frac{\Sigma(s^2S)}{\Sigma(S)} - \left(\frac{\Sigma(sS)}{\Sigma(S)} \right)^2 - \frac{1}{12}. \quad (\text{XII})$$

The last term, as has been mentioned, is generally negligible. As computed σ^2/ω^2 should turn out to be not more than about one-sixth of the range of s -values recorded for S -values different from zero.

(3) Determination of the enlargement factor

The quantity σ^2 (centimeters squared, in terms of actual distance in the cell) is now computed from σ^2/ω^2 (in graph units squared) by multiplying by ω^2 after determining ω , the distance in the cell corresponding to one coordinate unit.

Measurement of the photographic enlargement factor from the cell to the plate has been described in section 1. The factor designated F is the distance, for example, in centimeters, in the cell corresponding to one centimeter on the plate. If a coordinate grid,

having n rulings per centimeter is used in contact with the photographic record as described, F centimeters in the cell correspond to n rulings (at any degree of enlargement) and $\omega = F/n$.

(4) Reference of the data to "zero time": final determination of $D_{2,0}$

Correction of the measured time for the effect of disturbances in forming the boundary has been discussed in section III8c. The zero time and best average diffusion constant, $D_{2,0}$ can be computed in exactly the same way using $\sigma^2/2$ in place of $\omega^2 A^2/4\pi H^2$ as the function of time.

$D_{2,0}$ may be computed from individual values as $\sigma^2/2t$ using the corrected time in the computation; or it may be computed from pairs of values as $(d/dt)(\sigma^2/2)$, that is $(\sigma_2^2 - \sigma_1^2)/(t_2 - t_1)$ where σ_1^2 is the measured value at t_1 , σ_2^2 is the measured value at t_2 , and t_1 and t_2 may be taken as measured.

The diffusion constant may be stated as the number of grams of solute crossing a boundary of area one square centimeter in one second under a concentration gradient of one gram per cubic centimeter per centimeter in the direction of diffusion. Examination shows that this quantity reduces to units of centimeters squared per second.

The quantity σ found in the computation is the distance (corresponding to *two* positions in the cell) from the original boundary at which the concentration gradient has its root-mean-square value. In an ideal diffusing system with a single diffusing component, σ is the distance (the square root of the average squared distance) through which a representative molecule has moved in a given time (compare Fig. 3).

e. The diffusion of mixtures: "shape analysis" of the diffusion curve

As a consequence of the method of computation, the value $D_{2,0}$ of a mixture is a weight-average diffusion constant. This means that if compound A has a diffusion constant D_a and component B one of D_b , and these components are present in the concentrations a and b grams per ml., $D_{2,0}$ for the mixture will be $(a \cdot D_a + b \cdot D_b)/(a + b)$ and similarly for any number of components. It has the additional advantage that it is less affected by random errors in analyzing the diffusion curve than other averages which can be computed.

The diffusion curve of a homogeneous, ideally diffusing substance, has the shape of the normal curve of error or probability curve. Diffusion curves of a homogeneous, ideally diffusing substance diffusing from a boundary not perfectly sharp initially approach the same shape as the changes due to diffusion become more important than the initial disturbance. Curves of mixtures of substances on the other hand, can be readily shown to give curves which are more pointed than the normal curve. As a consequence, $D_{0,h}$, of an ideal mixture is always smaller than $D_{2,0}$ because of the way the accentuated height enters into the computation of the former. In fact, the ratio of $D_{2,0}$ to $D_{0,h}$ is a convenient index of non-uniformity, being unity for homogeneous ideal substances. For careful work, values of the ratio should be computed for several records near the end of the analysis to determine any trend due to the smoothing out of an anomalous shape introduced at the formation of the original boundary. Also, one should use values of $D_{0,h}$ computed using $\Sigma(S)$ as the area, rather than that found with a planimeter or by weighing in order to remove any discrepancy from this source. It will be noticed that neither the time nor the enlargement factor enters into this comparison.

The computations can be summarized as follows:

$$D_{0,h} = \left(\frac{\Sigma(S)}{S_{\max}} \right)^2 \cdot \frac{\omega^2}{4\pi t} \quad (\text{XIII})$$

$$\frac{D_{2,0}}{D_{0,h}} = \frac{2\pi [\Sigma(s^2S)\Sigma(S) - \Sigma^2(sS)]}{S_{\max}^2} \quad (\text{XIV})$$

In some instances skewed (62) or flattened diffusion curves have been encountered; these have usually been qualitatively accounted for by special properties of the diffusing material or of the conditions of diffusion.

The most detailed comparison of a diffusion curve with that of an ideal homogeneous substance is made by normalizing the experimental curve. This means multiplying the coordinates of the experimental curve, the s - and S -values, by factors such that the standard deviation and the area both become unity, as in the normal curve of error; and then comparing the experimental curve in normal coordinates with the normal curve. The transformation makes use of the following relations.

Normalized abscissa:

$$\xi = \frac{(s - \beta)\omega}{\sigma} \quad (\text{XV})$$

(This also moves the axis of ordinates to its true position giving the smallest value for the second moment.)

Normalized ordinate:

$$\Xi = \frac{\sigma S}{\omega \Sigma(S)} . \quad (\text{XVI})$$

The normalized coordinates are then plotted on the same graph as the normal curve of error, giving an immediate indication of any anomaly present.

f. Reference of diffusion constants to standard conditions

The diffusion constant may be determined under various experimental conditions. For purposes of comparison it is desirable to give results referred to standard conditions and to be able to compute equivalent values for other experimental conditions. Diffusion constants are commonly referred to pure water at 20°. (Occasionally 25° or 0° is used as a standard temperature.)

Three factors are taken into account: the direct effect of temperature change on the thermal motion of the solute, affecting the diffusion constant in direct proportion to the absolute temperature; the effect of temperature on the viscosity of the medium used; and the effect of the buffer salts or other components of the solvent on the viscosity of pure water. Explicitly, if $D_{w,20}$ is the diffusion constant in water at 20°, $D_{b,t}$ is the diffusion constant in the buffer used at the experimental temperature t° (centigrade), $\eta_{b,20}$ is the coefficient of viscosity of the buffer at 20°, $\eta_{b,t}$ is its coefficient of viscosity at t° , and $\eta_{w,20}$ is the coefficient of viscosity of water at 20°:

$$D_{w,20} = D_{b,t} \times \frac{273.2 + 20}{273.2 + t} \times \frac{\eta_{b,t}}{\eta_{b,20}} \times \frac{\eta_{b,20}}{\eta_{w,20}} , \quad (\text{XVII})$$

or simply

$$D_{w,20} = D_{b,t} \times \frac{293.2}{273.2 + t} \times \frac{\eta_{b,t}}{\eta_{w,20}} . \quad (\text{XVIII})$$

In order to illustrate the evaluation of the viscosity factors, we cite as an example a diffusion constant 9.46×10^{-7} cm² second⁻¹ measured at 28.8° in sodium phosphate buffer containing Na₂HPO₄:0.0194 M, NaH₂PO₄:0.0417 M, which is to be referred to the medium pure water at 20°.

(1) The immediate effect of temperature change from 28.8° to 20° is to reduce the kinetic energy of the molecules by the factor 293.2/302.0.

Correction of the viscosity for temperature change and for salt content may be done by measurement of the viscosity of the buffer at the temperature of the diffusion experiment, taking the viscosity of water at the standard temperature from published tables, and applying the indicated correction. However, in some instances it is also possible to estimate the viscosity correction from published viscosity data in two steps as will be done in the following paragraphs.

(2) The diffusion constant at 20° will be less than that at 28.8° in the ratio of the viscosity of the media at the two temperatures. The effect of temperature on the viscosity of the buffer is taken as essentially the same as its effect on the viscosity of water, which is easily available in tables; namely, at 20° the viscosity of water is 1.0050 centipoises and at 28.8°, by interpolation, 0.8216 centipoise (8) giving the relative viscosity 0.8175.

(3) The effect of buffer salts on the viscosity of the medium is such that the diffusion constant in pure water will be greater than that in salt solution in the ratio of the viscosities of the salt solution and the water at the given temperature. Appendix III, Svedberg and Pedersen (90), gives the following relative viscosities:

Concentration	NaH ₂ PO ₄ 20°	Na ₂ HPO ₄ 30°
M/100	1.003	1.007
M/10	1.030	1.066

The temperature coefficient of the *relative* viscosity is probably negligible (as it is for NaCl). One assumes that for such dilute solutions the increments in relative viscosity are additive and finds, for the buffer used:

Relative viscosity of water:	1.0000
Increment due to 0.0194 M Na ₂ HPO ₄ = 0.0194 × 0.66 = 0.0128	
Increment due to 0.0417 M NaH ₂ PO ₄ = 0.0417 × 0.30 = 0.0125	
Viscosity of buffer relative to that of water	<u>1.0253</u>

The diffusion constant referred to the medium, water at 20° is therefore

$$9.46 \times 10^{-7} \times \frac{293.2}{302.0} \times 0.8175 \times 1.025 = 9.46 \times 10^{-7} \times 0.8138$$

$$= 7.70 \times 10^{-7} \text{ cm.}^2 \text{ sec.}^{-1}.$$

Diffusion constants are known to depend upon concentration; and in fact the dependence is due to the same causes as the depend-

ence of the reduced osmotic pressure and the sedimentation constant upon concentration, and may be summarized by an activity coefficient, for the solute, of less than unity. For this reason, diffusion constants are measured in as dilute solution as convenient. Where desirable, measurements may be made at several concentrations and the extrapolated value of the diffusion constant at zero concentration estimated, to find the true diffusion *constant* characteristic of the system apart from interaction effects.

g. Estimation of molecular weight from diffusion constants

Under certain assumptions, including the absence of interaction effects, sphericity of the diffusing molecules, and size of the solute molecules very much larger than that of the solvent molecules so that the solvent is essentially without structure, the Stokes-Einstein (23) relation may be used to estimate the size and molecular weight of the diffusing kinetic unit:

$$D = \frac{RT}{6\pi\eta Nr} \quad (\text{XIX})$$

D = diffusion coefficient ($\text{cm.}^2 \text{ second}^{-1}$) $R = 8.3143 \times 10^7 \text{ erg degree}^{-1} \text{ mole}^{-1}$ (gas constant)

T = temperature in degrees Kelvin (absolute)

η = coefficient of viscosity of the medium (poise)

$N = 6.0228 \times 10^{23} \text{ mole}^{-1}$ (Avogadro's number)

r = radius of the molecule (centimeter)

For our purpose, it will be convenient to recall that the weight of N spheres of radius r is

$$\frac{4\pi N \rho r^3}{3} = M \quad (\text{XX})$$

where ρ is the density of the solute (grams per cm.^3) and M is the molecular weight. From this relation,

$$M = \frac{R^3 T^3 \rho}{162 \pi^2 \eta^3 N^2 D^3} \quad (\text{XXI})$$

As an instructive example, the molecular weight of pentaerythritol may be estimated from its diffusion constant, $6.80 \times 10^{-6} \text{ cm.}^2 \text{ second}^{-1}$ in water at 20° at $0.05 M$, cited by Lamm (48). In this instance $T = 293.18$, $\rho = 1.548$, and $\eta = 0.010050$ poise, giving $M = 121$ for comparison with the formula weight 136.

Various constant factors have been proposed to replace the 6 of the Stokes-Einstein equation, particularly to extend its validity to

systems which do not fulfill the theoretical requirements. (Glasstone, Laidler, and Eyring (35) suggest that the constant probably will not be greatly different from unity in the diffusion of large molecules in a solvent consisting of small molecules.)

In the event that the diffusing molecules are known to deviate from behavior as spheres and if the appropriate frictional resistance ratio, f/f_0 , is known (87, 90), for example from viscosity or sedimentation velocity measurements, the molecular weight computed for spherical molecules may be corrected by dividing by the cube of this ratio. This correction may also be made implicitly, the ability to eliminate the evaluation of frictional resistance factors being an important advantage of introducing ultracentrifugal methods.

IV. SEDIMENTATION ANALYSIS

1. The Purpose of Sedimentation Measurements

Ultracentrifugal analysis aims to determine the sedimentation constant characterizing a given molecular species by measuring the sedimentation velocity. From this, the molecular weight can be computed if the diffusion constant is known, or if the molar frictional coefficient is known from other data such as viscosity. By the sedimentation equilibrium method, molecular weight can be measured directly.

In addition, sedimentation analysis can show the stability and purity of a protein. A homogeneous protein, of high molecular weight, sediments with a sharp, symmetrical boundary in an ideally applied high centrifugal field. Stability properties are shown by comparing sedimentation in media of various pH's, protein concentrations, or with specific reagents such as urea.

2. The Principles of Measurement

The sedimentation velocity and equilibrium methods differ fundamentally. In the first, the sedimentation rate is measured, divided by the magnitude of the centrifugal field used, and referred to standard conditions to give the sedimentation constant. The centrifugal field is great enough that diffusion, which spreads the sedimenting boundary, does not prevent accurate measurement. The equilibrium method uses a lower centrifugal field. The molecular weight is found from the distribution of solute.

Both methods require measurement of *concentration gradients*, discussed in detail on pages 332–334. Sedimentation velocity is measured by the rate of movement of a gradient with a maximum value

through the column of solution centrifuged. At equilibrium, the gradient is greatest at the "bottom" of the cell, decreasing regularly to the surface; the molecular weight is found by the manner of decrease.

The derivation of molecular weights from ultracentrifugal data is outlined below in accordance with Svedberg (90).

Sedimentation equilibrium. Sedimentation and diffusion reach equilibrium in a solution in a closed cell centrifuged long enough. At equilibrium, the increment, ds , of solute driven in time dt by the centrifugal force in the direction of the periphery through unit surface is the same as the increment moved by diffusion in the opposite direction. The increment for sedimentation is

$$ds = \frac{c\omega^2 x M(1 - V\rho)dt}{f} \quad (\text{XXII})$$

and for diffusion

$$ds = -\frac{RT}{f} \frac{dc}{dx} dt. \quad (\text{XXIII})$$

In these equations, R is the gas constant, M the molecular weight of the solute, T the absolute temperature, f the molar frictional coefficient, ω the angular velocity of the centrifuge, V the partial specific volume of the solute, ρ the density of the solvent, c the concentration of the solute, and x the distance from the center of rotation. Equating expressions (XXII) and (XXIII) and collecting variables gives

$$\frac{dc}{c} = -\frac{M(1 - V\rho)\omega^2 x dx}{RT}. \quad (\text{XXIV})$$

Integration between two distances, x_2 and x_1 , from the center of rotation then gives

$$M_c = \frac{2RT \ln (c_2/c_1)}{\omega^2(1 - V\rho)(x_2^2 - x_1^2)}. \quad (\text{XXV})$$

Sedimentation velocity. After a short initial period, a solute in a centrifugal field sediments with a centrifugal force per gram mole, $M(1 - V\rho)\omega^2 x$, exactly opposed by the frictional force per gram mole, $f dx/dt$. The meaning of the symbols has been given. The molar frictional coefficient f is conveniently found from the diffusion constant D as

$$f = \frac{RT}{D}. \quad (\text{XXVI})$$

The molecular weight from sedimentation velocity and diffusion measurements may therefore be given as

$$M_{sD} = \frac{RT}{D(1 - V\rho)} \frac{dx/dt}{\omega^2 x} . \quad (\text{XXVII})$$

The sedimentation velocity per unit field, $(dx/dt)/\omega^2 x$, characterizes a molecular species in a given medium at a given temperature. It is the specific sedimentation velocity or sedimentation constant and is denoted by s . Therefore

$$M_{sD} = \frac{RTs}{D(1 - V\rho)} . \quad (\text{XXVIII})$$

The centrifugal field varies directly with distance from the axis of rotation. When the observed boundary displacement in an interval is small compared with its distance from the axis, the displacement and the average position of the boundary may be used to estimate dx/dt and the average centrifugal field, and s computed as in Table V. For larger relative displacements s should be checked using the relation

$$s = \frac{\ln (x_2/x_1)}{\omega^2 (t_2 - t_1)} . \quad (\text{XXIX})$$

derived by integration.

These derivations of molecular weight from sedimentation data are as unrestricted as to the solvation and shape of the molecules as any classical method. Electric charge affects sedimentation, but its effects are known and easily avoided by working near the isoelectric point or adding sufficient salt. The derivation of the molecular weight from sedimentation velocity and diffusion assumes that the molar frictional coefficient f is the same for sedimentation

$$f = \frac{M(1 - V\rho)}{s} \quad (\text{XXX})$$

as for diffusion

$$f = \frac{RT}{D} . \quad (\text{XXVI})$$

It is reasonably supposed that shape and solvation affect f in the same way in each instance, so that it can either be eliminated, for computing molecular weight, or specifically evaluated.

Since at equilibrium between diffusion and sedimentation there

is no net transfer of material, equation XXV can also be derived thermodynamically and the molecular weight found quite independently of the molar frictional coefficient, which can then be found from equation XXX, using the sedimentation velocity, or from diffusion (equation XXVI) alone.

The molecular weight computations derived make no assumptions as to shape or solvation. However, a provisional estimate of the molecular weight may be made from the sedimentation constant alone, just as from the diffusion constant alone, by assuming the molecules to be unsolvated spheres and deriving the corresponding molar frictional coefficient

$$f_0 = 6\pi\eta N \left(\frac{3MV}{4\pi N} \right)^{1/3}, \quad (\text{XXXI})$$

from Stokes' law

$$\nu = \frac{2r^2g(\rho_p - \rho_l)}{9\eta}. \quad (\text{XXXII})$$

In this, ν cm. sec.⁻¹ is the velocity of movement of a particle of r cm. radius and density ρ_p g. cm.⁻³ in a gravitational or centrifugal field of g cm. sec.⁻². In a medium of density ρ_l g. cm.⁻³ and viscosity η poises.

Equating equations XXVI and XXXI yields equation XXI, already used to compute a molecular weight that is the greatest possible for a substance having a given diffusion constant. Similar combination of equations XXX and XXXI gives

$$M^2 = \frac{162\pi^2 N^2 \eta^3 V_s^3}{(1 - V\rho)^3}; \quad (\text{XXXIII})$$

this molecular weight is the least possible for a substance of given sedimentation constant. It is supposed that no molecule of given mass can sediment nor diffuse more quickly than an unsolvated sphere. The effect of charge being minimized experimentally, the true molecular weight will lie between these extremes. It is found as described from equations XXVIII or XXIX without invoking Stokes' law.

The ratio of the measured molar frictional coefficient to that given by equation XXXIII for unsolvated spheres of the same mass is a convenient measure of the combined effect of solvation and shape. These factors may be resolved by methods described in *The Ultracentrifuge* (90) and by Oncley (71).

3. Apparatus for Sedimentation Analysis

Required for sedimentation analysis is a means of subjecting solutions to accurately controlled centrifugal fields, more specifically, in an optical cell of a rotor which can operate free from vibrations and fluctuations in temperature. For a long time the only instruments available capable of meeting these requirements were the two Svedberg centrifuges, the oil-turbine ultracentrifuge designed principally for sedimentation velocity measurements and the electrical centrifuge for sedimentation equilibrium studies (90). In recent years in this country several ultracentrifuges have been introduced and developed based on different driving principles, but employing rotors, cells, and optical systems like those of the Svedberg instruments (3, 4, 5, 6, 7, 43, 58, 59, 75, 78, 96). We shall summarize briefly some features of the various centrifuges.

The essential part of the oil-turbine ultracentrifuge is the elliptically shaped chrome nickel steel rotor which operates on horizontal bearings, driven by a stream of high speed oil impinging upon small vanes located on each end of the horizontal shaft. The rotor is enclosed in a heavy steel housing as a safety measure. Hydrogen gas at low pressure is circulated between the housing and the rotor for temperature control. The rotor accommodates two cells made of duralumin; one of these is the analytical cell, having a wedge-shaped cavity for the solution. This cell is fitted with quartz windows on each end to permit light to pass for the recording of sedimentation. The center of the cell is 6.5 centimeters from the axis of rotation and the column of solution is 1.8 centimeters high. The other cell is the balancing cell placed in a hole on the opposite side of the rotor and is required to maintain static and dynamic balance necessary for vibration-free operation. The balancing cell serves another purpose: it contains a small hole, called the index, which permits light to pass to form a record on the photographic plate together with the sedimentation record. The record from the balancing cell is a reference point from which the distance of the midpoint of the sedimenting boundary to the center of rotation is determined, the hole in the balancing cell being at a known distance from the center of rotation for all speeds of the rotor. The combined arrangements employed for the measurement and control of temperature, lubrication of bearings, and driving speed in the oil-turbine instrument give a stable instrument with which satisfactory results in sedimentation analysis are obtained routinely with maxi-

imum speeds of as much as 80,000 revolutions per minute (maximum field of about 500,000 g).

The Svedberg equilibrium centrifuge is a much simpler instrument; it is designed for use in place of the oil-turbine ultracentrifuge when lower centrifugal fields are required, as for equilibrium measurements. A steel rotor is employed which is cylindrical in shape. It operates on a vertical shaft which eliminates the stringent requirements for static and dynamic balance demanded by the horizontally driven oil-turbine motor. It is powered by direct connection with a synchronous electric motor, and operates to a maximum speed of 18,000 revolutions per minute. The analytical cell and its balancing cell are similar in design to the cells used in the higher speed instruments. The center of the analytical cell is 5.75 centimeters from the center of rotation and the solution column is 0.5 centimeter high.

The new ultracentrifuges differ principally in the driving mechanism, employing air-driven or electrically driven tops. The simplest of these new instruments, useful for very limited applications, is the McBain air-driven spinning top ultracentrifuge (58, 59). The analytical cell is placed in the top itself; because of the small radius and the short cell column the centrifugal field developed in the cell is highly inhomogeneous, an undesirable feature of this instrument. Other ultracentrifuges, developed principally by Beams and Pickels employ a spinning top to drive a dural rotor of size comparable to the oil-turbine rotor. The rotor, Fig. 14, operates on a vertical drive, being attached to the driving top by a steel piano wire. The analytical cells and the balancing cells are similar in design to the Svedberg cells and are located at a similar distance from the axis of rotation. The maximum speeds of operation are 60,000 to 70,000 r.p.m. An electrically driven ultracentrifuge using a dural analytical rotor capable of routine use at 60,000 r.p.m. is now available commercially (78).

For further discussion of the mechanical details of various ultracentrifuges, the student is referred to the Svedberg and Pedersen monograph, *The Ultracentrifuge* (90) and to other excellent reviews (66, 71, 76, 77, 93).

4. Measurement of Sedimentation Velocity and Equilibrium

The sedimentation velocity and equilibrium methods are similar in certain respects; both consist in enclosing the solution to be



FIG. 14. Rotor of Beams-Pickels air turbine ultracentrifuge showing the analytical and balancing cells.

studied in an analytical cell, accelerating the cell in a rotor to the desired speed, and measuring the displacement of the sedimenting solute. Both employ the optical methods for analyzing sedimentation boundaries described, beginning page 339, for the measurement of diffusion. Similar requirements hold for the solution to be studied.

a. Protein solution

The solution of protein for sedimentation analysis is made up together with a salt and a buffer of desired pH. The range of concentration of protein which can be studied is limited by the present optical methods. Below 0.2% the methods are not sufficiently

sensitive and above 3% to 4% anomalous optical effects begin to appear which render measurements difficult. For molecular weight determination the concentration of the sedimenting species should be kept below 1%. It may be necessary to study a range of concentration (see page 331). The presence of low molecular weight electrolyte is essential to overcome the retarding effect of the tendency for centrifugal separation of particles of widely different mass and opposite charge when the protein is not near its isoelectric point. For a one per cent protein solution not near its isoelectric point the effect may usually be offset by the presence of 0.2*N* salt (90, page 26). Secondary effects may be avoided by using salts such as NaCl or KCl having ions of similar mass.

b. Centrifuge cells

The analytical cells are provided with an opening on the side for filling. Generally less than 1 ml. of solution is required, the amount varying with the thickness of the cell, which may be from 1.5 to 12.5 mm., the wider ones being suited for study of the more dilute solutions. With compensations in the optical enlargement a relatively wide range of concentration can be studied in the same cell.

A first consideration in making a sedimentation run is cleanliness of the cell. Under ideal conditions the cell should be dismantled following each run for cleaning the separate parts. If this procedure is not desirable, because of difficulty of assembly, the cell should be very carefully rinsed with the solvent and stored with water or a non-corrosive solvent. This is left in the cell between runs. Before a run the cell is rinsed with some of the protein solution to be analyzed.

The solution in the cell must be adequately covered to prevent evaporation of solvent into the evacuated chamber during the run. Otherwise, convection currents may make optical analysis of sedimentation difficult or impossible. Either a snugly fitting lid or a layer of paraffin oil can be used to seal the cell opening.

Before placing the cell in the rotor, a check is made to insure that the cell windows are clean and dry on the outside; water, oil, or particles of lint can seriously interfere with the optical measurements. The cell is placed in the rotor, with its bottom toward the periphery of the rotor and its vertical axis accurately in line with the axis of rotation. Reference marks on the cell and rotor are provided for this purpose. The appropriate balancing cell is placed in the hole opposite the analytical cell; it is lined up so that the index is toward the periphery.

Details for driving the different centrifuges vary. Since, as a rule, directions accompany the individual instrument, it will not be necessary to describe them here.

c. Sedimentation velocity

(1) Speed requirements

Because the speed suitable for a sedimentation velocity run will depend not only on the size, shape, and density of the sedimenting species, but also on the density and viscosity of the medium, no hard and fast rule can be laid down regarding the speeds to be used for the analysis of a protein of known size. In general, speeds of 50,000 to 60,000 r.p.m. are suitable for proteins having molecular weights up to 200,000 to 300,000 and correspondingly lower speed for proteins of higher molecular weights. The rate of sedimentation should not be so rapid as to get blurred photographs resulting from appreciable sedimentation during the time required for an exposure. On the other hand, the rate should be high enough to obviate the obscuring effect of diffusion. Generally a sedimentation rate is preferred which allows at least five to eight exposures with 10 to 20 minute intervals between each, with a reasonably well-defined boundary over the period of the run.

When the size or sedimentation characteristics of the protein are entirely unknown, preliminary observation of the sedimentation is necessary. For this it is customary to accelerate the rotor slowly and to watch for indications of a sedimenting boundary and its rate of movement as the speed is increased. Persistence of vision permits an apparently continuous view through the rotating cell so that with the schlieren optical methods it is relatively easy to detect the presence of a sedimenting boundary.

When the scale method (page 340) is used, the scale itself or its image is placed in a position that will give maximum enlargement of the gradient in the cell, for example at a scale distance of 10 or 12 centimeters. The appearance of a boundary will cause a displacement of the scale lines which is observed at the position of the photographic plate by means of a short focal length lens. As sedimentation continues, the blurred region of the scale will be seen to move apparently toward the center of rotation, but actually away from the center because of the inversion of the image by the camera objective. It is important to recognize that scale line displacement can be caused by effects other than by a sedimentation gradient, for example, by convection currents or, in case of leakage, by the liquid surface. Cell leakage can also result in throw-

ing off the rotor balance sufficiently to cause vibration. Whenever this condition is indicated, the run is immediately shut down.

As soon as the sedimentation boundary is well in the optical field the rotor acceleration is discontinued, the speed is adjusted to a constant value, and the rate of sedimentation is observed. Further adjustment of the speed may be required until the rate of sedimentation is suitable for measurement.

(2) Experimental record

The relationship of the centrifuge rotor to three optical methods of recording the movement and size of sedimenting gradients is illustrated in Figure 15. In recording sedimentation, the photographic enlargement is adjusted to give a large displacement of the boundary

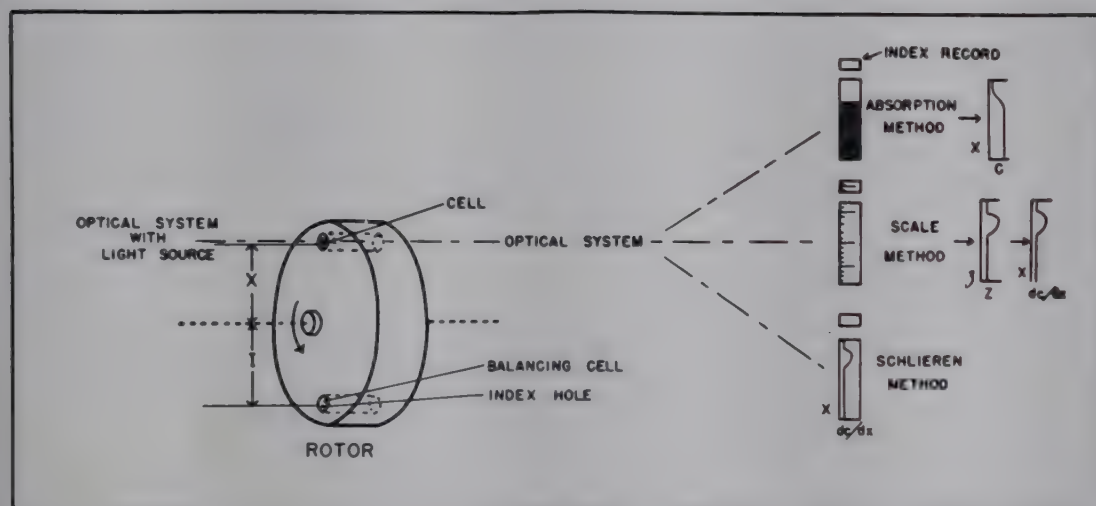


FIG. 15. Relationship between mechanical and optical systems in the ultracentrifuge.

record to be useful for subsequent computations. To compensate for boundary spreading during the course of a run, the enlargement is increased correspondingly. In using the scale method, the enlargement of the boundary is made by adjustment of the position of the scale (or the scale image, whichever is used) with reference to the cell. The scale must always be in focus on the camera plate. The camera settings for various scale positions are a part of the permanent calibration of the instrument. A suitable small aperture, as from $f:40$ to $f:60$, is used in the camera to give good definition of all scale lines.

In using the schlieren method, the enlargement of the boundary is affected as described on page 358, by the angle of the diagonal slit. In this case, too, the enlargement employed should be large but for ease of measurement, not greater than that which gives a sharp pattern.

Exposures are taken until the boundary is three quarters of the distance through the column; beyond this point the sedimentation rate may be influenced by the effect of increasing solute concentration at the bottom. During the run a record is kept of the exposure intervals, optical settings, and the temperature and speed of the rotor.

After the last exposure is taken, the centrifuge is shut down, the cells are removed and cleaned, and the photographic plates are developed using a high contrast developer recommended by the manufacturer of the plate used. (See discussion on photography of refraction gradients, page 352.)

With the scale method, an additional run is made with the solvent alone. In this reference run, the scale is photographed under conditions identical with those used in the principal run. This procedure is used in order to eliminate the effects on the scale positions of hydrostatic pressure, distortion of the cell and windows in the high field, as well as the slight, but appreciable sedimentation of the buffer and salt. By subtracting the scale records of the two runs, scale line displacement is obtained due only to the sedimentation of the protein solute.

Several precautions are taken in making the reference run. If the composition of the solvent differs from that used in the principal run, the resulting sedimentation displacement curve may have a slanting base line. Furthermore, should the speeds of the two runs differ by more than about 2000 r.p.m. the base line may be shifted from zero as well as being slanted. Slight errors in the base line, although not critical for the determination of the sedimentation constant, must be taken into account when the concentrations of sedimenting species are to be measured.

(3) Computation of sedimentation constant

The standard terminology used in sedimentation analysis is given in Table IV.

The following data are necessary or convenient.

1. Record of the times of photographic exposures made during sedimentation.
2. Record of operating conditions including the temperature of the rotor, optical settings, and rotor speed.
3. Chart giving the distance of axis of rotation to the center of index as a function of the speed of the rotor. This may not be needed with some rotors.
4. Charts of the density increment with concentration for various buffers and salts used (90).

TABLE IV
NOTATION USED IN DISCUSSING SEDIMENTATION

a cm.: the length of the optical path through the centrifuge cell contents; or half the length of the long axis of a prolate (elongated) ellipsoid of revolution.

b cm.: the optical distance between the middle of the cell and the scale, or scale image. The optical distance is the sum of the distances traversed by light through the various media, each distance being divided by the refractive index of the corresponding medium. Or *b* may denote one-half the length of the short axis of a prolate ellipsoid of revolution.

c moles per liter: solute concentration at positions indicated by subscripts.

D (subscript): "determined from the diffusion constant."

f: molar frictional coefficient, the hydrodynamic resistance offered to movement of one mole of solute per unit rate of displacement.

f₀: the molar frictional coefficient of a solute having its molecules in the form of solid, anhydrous spheres.

f/f₀, the molar frictional ratio, is analyzed into two factors, *f/f_e*, accounting for the effect of hydration on the molar frictional coefficient, and *f_e/f₀*, accounting for the effect of shape.

F cm.: the distance in the cell corresponding to one unit of *z*.

G: the number of units of *z* corresponding to one cm. on the scale. $G = (l - b)/Fl$.

H₂O (subscript): "in a medium having the density and viscosity of pure water."

I cm.: the distance from the axis of rotation to the center of the index hole, or equivalent reference point.

l cm.: optical distance from camera objective to middle of cell.

ln: the natural logarithm, to the base $e = 2.7183$, of the term following.

m gm.: weight of a pycnometer with a solution of weight fraction indicated by a subscript.

m (subscript): "average."

M: molecular weight, the method or conditions of estimation being indicated by subscripts.

n: refractive index.

p: comparator reading for middle of index hole or equivalent reference point.

q: comparator reading of selected reference line image.

r: grams of water bound per gram of protein in solution.

R: molar gas constant, 8.3143×10^7 ergs per degree per mole.

r.p.m.: revolutions per minute.

s seconds: specific sedimentation velocity in unit centrifugal field with unit density difference, the sedimentation constant, the conditions of estimation being given by subscripts. In particular, *s₂₀* is the sedimentation constant referred to a medium of the density and viscosity of water at 20°.

s (subscript): "as determined from sedimentation velocity" or "as measured in the solvent used."

S: unit of sedimentation constant, the Svedberg, 10^{-13} second.

t seconds: time corresponding to observed position of sedimenting boundary indicated by subscript; with subscript *m*, the average solution temperature in an interval of measurement.

t (subscript): "as measured at the experimental temperature."

T °K: absolute temperature; degrees Centigrade plus 273.2.

V cm.³ per gm.: the partial specific volume of a component, indicated by a subscript, of a system; with superscript *a*, the apparent partial specific volume, the observed change in the volume of a solution per gram of added solute at finite concentrations.

v cm.³: volume of pycnometer.

w: weight fraction of solute in solution corresponding to pycnometer weight indicated by numerical subscript; with subscript *p*, weight fraction of solute.

x cm.: distance of refractive gradient from axis of rotation; with subscript zero, corresponding to scale line position of corresponding subscript; with numerical subscript, corresponding to indicated time.

z: position of scale line image in arbitrary units of known size measured from an arbitrary zero a known distance from the axis of rotation.

Z: displacement of scale line image because of presence of a refractive gradient

20 (subscript): "as it would be observed at 20°."

Δ : indicating the difference of two values of the following quantity.

η *poise*: coefficient of viscosity, the conditions or composition indicated by subscripts.

π : 3.1416.

ρ *gm. per cm.³*: density, the conditions or composition indicated by subscripts.

ω radians per second: average angular rotational velocity in a given interval.

5. Charts of the relative viscosity with respect to water for various concentrations of the buffers and salts used (90).

6. Charts of density and viscosity of water at various temperatures.

7. Chart of ω^2 vs. r.p.m. $(\text{r.p.m.})^2 \times 0.010966 = \omega^2$ (radians per second)².

8. Table of magnification factors for camera including values for F and G for various optical settings.

The procedure for calculation of the sedimentation constant is the following:

1. Determination of the distance from the midpoint of the sedimentation boundary to the index on each photograph.

2. Conversion of the distance from the boundary to the index as measured on the plate to the actual distance in the centrifuge cell for each exposure.

3. Determination of the distance of the boundary from the center of rotation for each exposure.

4. Determination of the displacement of the boundary during each interval between exposures.

5. Calculation of the sedimentation constant for each exposure interval.

6. Correction of the average sedimentation constant from the experimental conditions to sedimentation in pure water at 20°.

The first step in computing the sedimentation constant by the scale line method is to measure the position of each scale line for each record of the principal run and of the corresponding reference run. A microcomparator or traveling microscope reading to one micron is used. Each record is placed on the stage so that the scale record is parallel to the line of travel of the instrument. Readings are usually made with reference to an arbitrary reference line, the same for each exposure, in the index record. This reference line is not necessarily the center of the index, but should be clearly defined in each exposure.

When the scale line positions have been measured for a record and its corresponding reference record, the difference between each pair of corresponding readings is found. These differences, Z, are the line displacements due to the sedimenting refractive gradient.

The Z value for each line is then plotted as ordinate against the comparator reading for the same scale line in the record of the sedimenting gradient. Such a graph, illustrated in Fig. 16, shows the refractive index gradient as a function of position in the cell, the coordinates being determined by known optical enlargement factors and by the units chosen for plotting.

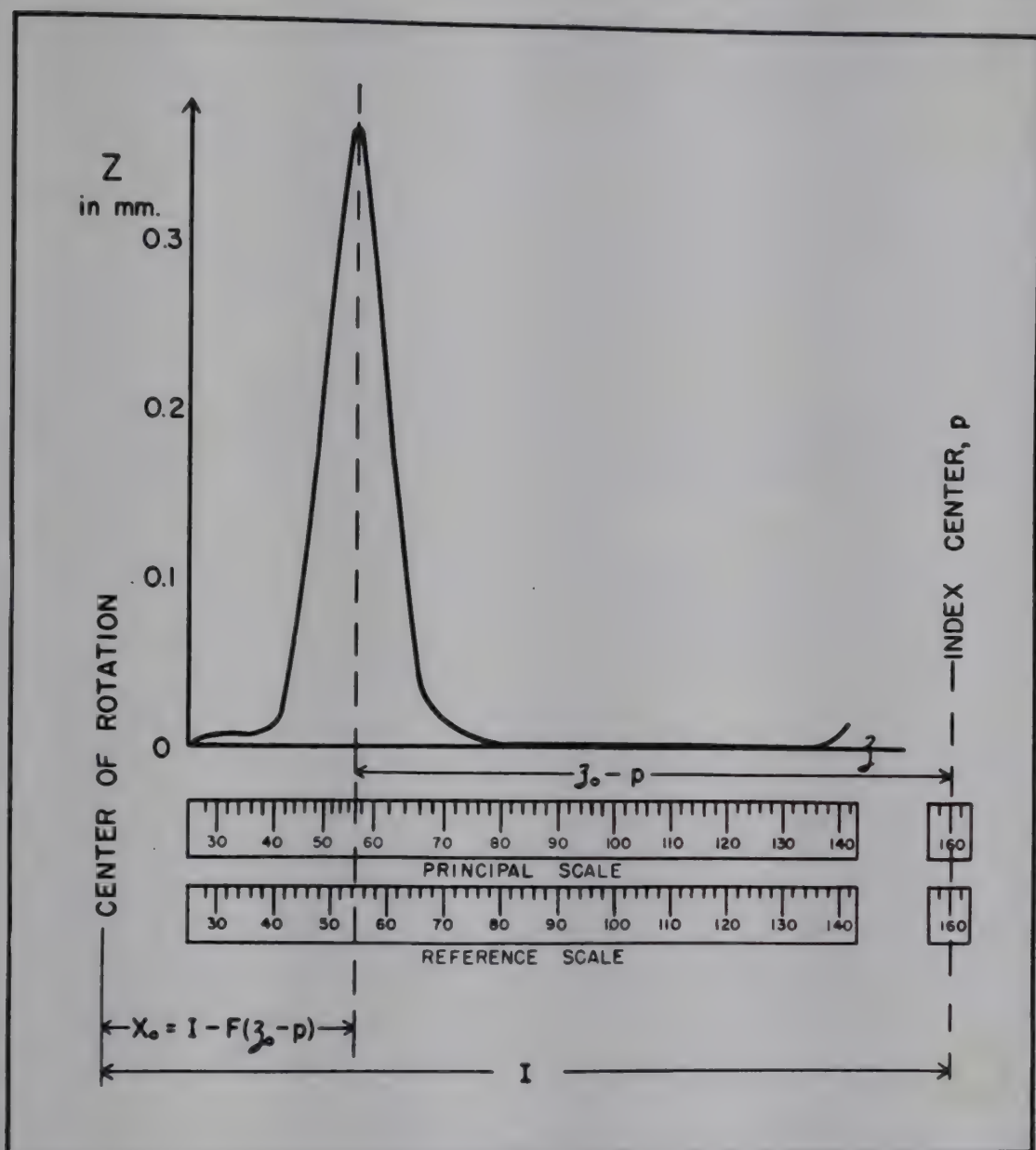


FIG. 16. Relationship between the scale line for a sedimentation velocity measurement displacement curve, the scale records, and the distances from the center of rotation in the rotor.

The position z_0 of the midpoint (modal point or maximum) of the gradient is next found in comparator units by reference to the graph. Ideally, the abscissa defined by the ordinate passing through the center of area of the plotted gradient curve gives a better defined, weight average value of the sedimentation of solute.

The scale of z being selected, as described, from an arbitrary reference line, the position, p , of the center of the index hole, is located on the same scale as follows. The reference line q is set under the comparator to give the same reading as in the measurement of the line displacements. The average of readings then made of both edges of the index gives p . A separate exposure made with a

small lens aperture may be made if necessary to define the edges of the index record more precisely. Fig. 16 shows the spatial relations. Where an edge of the index hole is well-enough defined, it may be used as the reference line instead of the center.

In this way an abscissa, z_0 , is found, directly from the graph, that defines the position of the sedimenting boundary in micro-comparator units. The distance, x_0 cm., from the axis of rotation to the boundary is given by the formula

$$x_0 = I \pm F(z_0 - p). \quad (\text{XXXIV})$$

I cm. is the distance from the axis of rotation to the center of the index at the speed of rotation used. F cm. in the cell correspond to

TABLE V

ILLUSTRATION OF COMPUTATION OF SEDIMENTATION CONSTANT

Protein: hog thyroglobulin.

Concentration: 0.7 gm. per 100 ml.

Buffer: NaCl: 0.1 M ; NaH_2PO_4 : 0.0133 M ; Na_2HPO_4 : 0.0019 M .

Exposure	Time	t sec.	x_0 cm.	x cm.	x_m cm.	r.p.m.	ω^2 $\times 10^{-5}$	s $\times 10^{13}$ sec.
1	2:05		6.002					
2	2:15	600	6.123	0.121	6.063	39,400	170.2	19.54
3	2:25	600	6.245	0.122	6.184	40,300	178.1	18.46
4	2:35	600	6.382	0.137	6.314	40,650	181.2	19.96
5	2:45	600	6.526	0.144	6.454	40,650	181.2	20.52
6	2:55	600	6.671	0.145	6.599	40,500	179.9	20.06
7	3:05	600	6.814	0.143	6.743	40,400	179.0	19.75
Average								19.7

For the interval between exposures 1 and 2:

$$s = \frac{(x_2 - x_1)}{(t_2 - t_1)\omega^2 x_m} = \frac{0.121}{600 \times 170.2 \times 10^5 \times 6.063} = 19.54 \times 10^{-13} \text{ sec.}$$

This is the observed sedimentation constant of this protein at the given concentration in the given buffer at the experimental temperature (23.26°).

one unit on the photographic record. z_0 and p are comparator readings defining the sedimenting boundary and index positions, as described. The algebraic sign before F is taken as plus when the record is measured so that comparator readings increase with distance from the axis of rotation or minus if the readings decrease as the distance from the axis increases.

When the schlieren method is used to record sedimentation, the refractive index gradient is recorded directly on the camera plate. Computation of the distance from the gradient in the centrifuge cell to the axis of rotation is exactly analogous to that just described for the scale method. The distance on the plate from the peak to the index record is measured. This distance is multiplied

by the appropriate reciprocal enlargement factor, F , to find the corresponding actual distance. This distance between the index and the gradient is then added to or subtracted from the distance of the index from the axis of rotation, as appropriate.

Computation of the sedimentation constant is illustrated in Table V, for an analysis of the protein hog thyroglobulin. The sedimentation rate is found from the change in position of the midpoint, x_0 , of the gradient in the interval between two exposures divided by the corresponding time. The sedimentation constant is this rate of sedimentation, in centimeters per second, divided by the average centrifugal field over this interval. Symbolically, the sedimentation rate is $(x_2 - x_1)/(t_2 - t_1)$ or $\Delta x/\Delta t$; the average centrifugal field is $\omega^2 x_m$, where ω is the average angular velocity of the rotor system in radians per second or revolutions per minute multiplied by $2\pi/60$ and $x_m = (x_2 + x_1)/2$ is the average position of the sedimenting boundary during the interval.

(4) Reference to standard conditions

To aid comparison of measurements made under various experimental conditions, it is convenient to report the estimated sedimentation constant a sedimenting species would have in a standard medium having the density and viscosity of pure water at 20° , even though direct measurement in such a medium may be impractical. The correction consists of several factors. One, usually computed separately for each interval, allows for differences in viscosity of the medium due to the difference of the temperature from 20° . In evaluating this factor, it is usually permissible to take the thermal coefficient of viscosity of dilute salt solutions to be the same as that of pure water. A second factor allows for the increment of viscosity due to dissolved buffer salts or other non-aqueous components of the medium of measurement. A third factor allows for the different buoyancies of protein in water and in the medium of measurement at any experimental temperature. A fourth factor allows for the different buoyancies of protein due to differences in its density and that of the medium at the experimental and reference temperatures. When the medium is a dilute aqueous buffer, its thermal coefficient of density may usually be taken as that of water. The last three factors are much less sensitive to temperature than the first. Therefore they are usually combined and used for all values in a given experiment. It is usually desirable to evaluate the first factor separately for each exposure interval. The total correction is:

$$\left[\frac{\eta_t}{\eta_{20}} \right]_{\text{H}_2\text{O}} \times \left[\frac{\eta_s}{\eta_{\text{H}_2\text{O}}} \right]_{t_{av.}} \times \left[\frac{1/V - \rho_{\text{H}_2\text{O}}}{1/V - \rho_s} \right]_{t_{av.}} \times \left[\frac{1 - V_{20}\rho_{20}}{1 - V_t\rho_t} \right]_{\text{H}_2\text{O}}$$

Here, η represents viscosity and ρ density, the temperature (20, t) and solvent (H_2O , s) being indicated by the subscripts. V is the partial specific volume of the protein. Application of these corrections is shown in Table VI.

(5) Partial specific volume

The partial specific volume of a solute is required for the reference of its sedimentation constant to standard conditions and also for the estimation of its molecular weight from the sedimentation and diffusion constants. The partial specific volume of a protein is approximately equal to the specific volume, the reciprocal of the dry density. More rigorously, it is the change in volume of the liquid that occurs when one gram of solute is dissolved in a large volume of solvent. The partial specific volumes of many proteins are near 0.75 cm^3 per gm. The value increases slowly with temperature, 0.0005 cm^3 per gm. per degree, and depends upon the composition of the protein. The partial specific volume of thymus nucleohistone is only 0.66 cm^3 per gm. because of its content of the denser nucleic acids. Lipoproteins have higher values because of the less dense lipids present. The partial specific volume of a protein can be computed from its amino-acid composition (19).

The partial specific volume is usually found by density measurement with a pycnometer of 5 to 20 ml. capacity (90, pp. 57–66). It is necessary to weigh to tenths of a milligram to determine the partial specific volume to within a few tenths of a percent. For many proteins V is nearly independent of concentration over a range of several percent. Therefore three or four consistent density determinations of solutions of various concentrations in a given solvent suffice. The apparent partial specific volume, V^a , is found from the formula

$$V^a = v \left[\frac{1}{m_0} - \frac{\left(\frac{1}{m_0} - \frac{1}{m} \right)}{w_p} \right] \quad (\text{XXXV})$$

in which $v \text{ cm}^3$ is the volume of a pycnometer, m_0 gm. its net weight filled with a solution of protein of weight fraction w_p , and m gm. its net weight filled with solvent at the same temperature.

When the partial specific volume varies with concentration, the net weight of the pycnometer contents may be plotted against the weight fraction of protein. For values sufficiently close together

TABLE VI

REFERENCE OF THE SEDIMENTATION CONSTANTS OF TABLE V TO STANDARD CONDITIONS. DATA FOR THE CORRECTION. THESE MAY BE DETERMINED EXPERIMENTALLY OR ESTIMATED AS SHOWN FROM PUBLISHED TABLES (90)

Component	Molarity	Viscosity Relative to Water (Near 20°) η_s/η_{H_2O}	Viscosity Increment $\Delta\eta$	Density gm. cm. ³ ρ	Density Increment $\Delta\rho$
Water		1.0000		0.9975 (23.26°)	
NaCl	0.100	1.0090	0.0090		0.0040
NaH ₂ PO ₄	0.0133	1.0040	0.0040		0.0013
Na ₂ HPO ₄	0.0019	1.0013	0.0001		0.0001
Buffer:		1.0143		1.0030	

$V_{10}=0.720$; $V_{21,30}=0.722$; $1/V_{21,30}=1.3850$.

Factor two, the viscosity of the experimental medium relative to that of water at the experimental temperature, is 1.0143, estimated above by adding the increments in relative viscosity due to the buffer salts, individually, to unity.

Factor three,

$$\left[\frac{1/V - \rho_{H_2O}}{1/V - \rho_s} \right]_{t_{av}} = \frac{1.3850 - 0.9975}{1.3850 - 1.0030} = 1.0144.$$

Factor four,

$$\left[\frac{1 - V_{20}\rho_{20}}{1 - V_t\rho_t} \right]_{H_2O} = \frac{1 - 0.720 \times 0.9982}{1 - 0.722 \times 0.9975} = 1.0054.$$

The product of factors two, three, and four is 1.034 and may be taken as constant for the given experiment.

Factor one; application of combined factors to data of table V.

Exposure	Observed Sedimen- tation Constant ($\times 10^{13}$ sec.)	Tempera- ture t_m°	Factor One $[\eta_t/\eta_{20}]_{H_2O}$	Combined Correction	Sedimen- tation Constant Referred to Water at 20°
1-2	19.54	23.26	0.9243	0.9562	18.68
2-3	18.46	23.65	0.9158	0.9474	17.49
3-4	19.96	24.00	0.9082	0.9395	18.75
4-5	20.52	24.15	0.9050	0.9362	19.21
5-6	20.06	24.20	0.9040	0.9352	18.76
6-7	19.75	24.27	0.9025	0.9336	18.44
				Average	18.56

Because the limiting experimental data: the measured displacement of the boundary between exposures and the partial specific volume factors, are given to only three significant figures, the sedimentation constant of hog thyroglobulin at 0.7 gm. per 100 ml. should be reported as 18.6×10^{-13} second or 18.6 S, referred to water at 20°, from the data given.

$(m_2 - m_1)/(w_2 - w_1)$ approximates the slope dm/dw_p , and the partial specific volume for a given concentration can be evaluated from the formula

$$V = v \left[1 - \frac{dm}{dw_p} \left(1 - \frac{w_p}{m} \right) \right]. \quad (\text{XXXVI})$$

(6) Extrapolation of the sedimentation constant to zero concentration

For some proteins the sedimentation constant varies appreciably with concentration because of interaction among the molecules in the solution. When the sedimentation constant, or in some instances the reciprocal of the sedimentation constant is expressed as a function of protein concentration, substantially a linear rela-

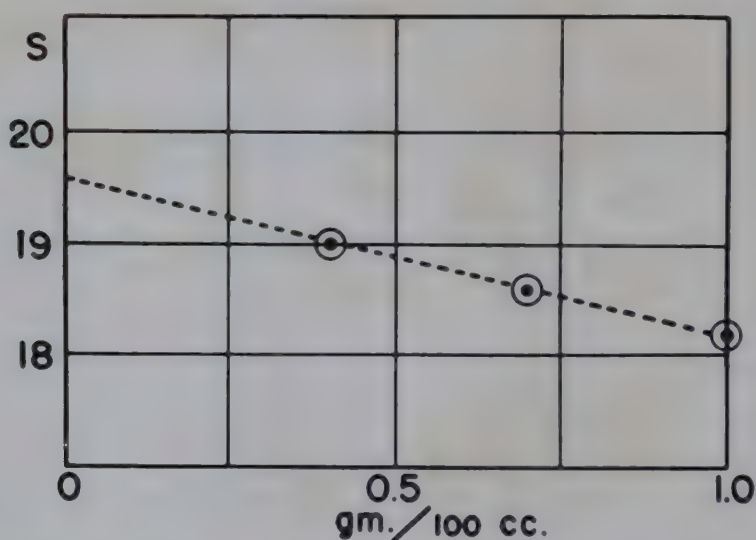


FIG. 17. Extrapolation of the measured sedimentation constants for hog thyroglobulin to infinite dilution. The extrapolated value, 19.6 S, represents the sedimentation free from interaction effects in solution.

tion is found. Linear extrapolation to zero concentration then determines a limiting sedimentation constant independent of interaction effects. Figure 17 shows this procedure for thyroglobulin, for which the extrapolated constant is 19.6 S.

The absolute unit for the sedimentation constant is the second. Most proteins have sedimentation constants between 10^{-13} and 150×10^{-13} second. Sedimentation constants are therefore commonly expressed in units of 10^{-13} second and it has been proposed to call this unit the Svedberg (symbol: S).

Sedimentation constants and other molecular data for a number of proteins are tabulated in Table VIII.

d. Molecular weight determination from sedimentation and diffusion

Molecular weights of sedimentable substances may be estimated

from their sedimentation and diffusion constants using Svedberg's relation

$$M_{sD} = \frac{RTs}{D(1 - V\rho)} \quad (\text{XXVIII})$$

in which M_{sD} is the molecular weight, R the molar gas constant, T the absolute temperature, s the sedimentation constant, D the diffusion constant, V the partial specific volume, and ρ the density of the medium. The quantities T , s , D , V , and ρ should all refer to the same experimental or reference medium at the same temperature. In this way the molecular weight of thyroglobulin is found from the data:

$R = 8.314 \times 10^7$ ergs per mole per degree

$s = 19.6 \times 10^{-13}$ second (20° water, zero concentration)

$D = 2.65 \times 10^{-7}$ cm.² per second (20° , water)

$V = 0.720$ cm.³ per gram (20°)

$\rho = 0.9982$ gm. per cm.³ (water at 20°)

$T = 293.2^\circ$ K. ($= 20^\circ$)

$$M_{sD} = \frac{8.314 \times 10^7 \times 293.2 \times 19.6 \times 10^{-13}}{2.65 \times 10^{-7} (1 - 0.720 \times 0.9982)} = 641,000.$$

This molecular weight, believed accurate to 5 to 10%, refers to the anhydrous protein, the partial specific volume being determined on the dry basis. Moreover, the molecular weight is not affected by assumptions as to molecular shape or the extent of hydration because sedimentation and diffusion presumably depend in the same way on these factors.

e. Relation of shape and hydration of molecules to their sedimentation and diffusion

The effect of shape and hydration of a molecule upon its sedimentation or diffusion is expressed as the molar frictional ratio of the solute. This ratio can be computed from the sedimentation and diffusion constants, taken together (90, pp. 38-44). The molar frictional ratio, f/f_0 , is the ratio of the resistance of a molecule to motion through a viscous medium in sedimentation or diffusion to the resistance of a spherical, anhydrous molecule of the same molecular weight. A spherical, unhydrated molecule therefore has the minimum molar frictional ratio, unity. Significantly, almost all proteins show frictional ratios between one and two, as in Table VII. The deviation from unity is due to the combined effect of solvation and molecular shape.

The frictional ratio can be found from the molecular constants

of a solute by either of the formulas:

$$\frac{f}{f_0} = 1.19 \times 10^{-15} \frac{(1 - V\rho)}{s} \left[\frac{M^2}{V} \right]^{1/3} \quad (\text{XXXVII})$$

$$\frac{f}{f_0} = \frac{2.89 \times 10^{-5}}{D(MV)^{1/3}} \quad (\text{XXXVIII})$$

$$\frac{f}{f_0} = \left[\frac{(1 - V\rho)}{D^2 s V} \right]^{1/3} \times 10^{-8}. \quad (\text{XXXIX})$$

M is the molecular weight of the solute. The quantities s , D , V , and ρ refer to the medium water at 20° in these instances.

For thyroglobulin:

$$\frac{f}{f_0} = \left[\frac{(1 - 0.720 \times 0.9982)}{(2.65 \times 10^{-7})^2 \times 19.6 \times 10^{-13} \times 0.720} \right]^{1/3} \times 10^{-8} = 1.42.$$

The molar frictional ratio may be considered (71) the product of two factors:

$$f/f_0 = f/f_e \times f_e/f_0, \quad (\text{XL})$$

such that the first, f/f_e , depends only on the solvation of the dissolved molecule and the second, f_e/f_0 , only on the molecular shape. The factor f/f_e is given as a function of hydration by Kraemer (46):

$$\frac{f}{f_e} = \left[\frac{rV_2 + V_1}{V_1} \right]^{1/3}. \quad (\text{XLI})$$

In this formula, r is the number of grams of water bound to each gram of protein, V_2 is the partial specific volume of water (1.00), and V_1 is the partial specific volume of the protein.

The factor f_e/f_0 can be related to the molecular proportions if one may assume, for example, that the molecules are essentially ellipsoids of revolution. Perrin (73) has shown the relation for prolate ellipsoids in terms of the ratio of the long to short axes, a/b :

$$f_e/f_0 = \frac{[1 - (b/a)^2]^{1/2}}{(b/a)^{2/3} \ln \left[\frac{1 + [1 - (b/a)^2]^{1/2}}{b/a} \right]}. \quad (\text{XLII})$$

Figure 18 shows this relation graphically. From it the value of a/b can be found when the ratio f_e/f_0 is known, for example from measurement of the double refraction of flow (22). The ratio f_e/f_0 may also be estimated from the measured molar frictional ratio,

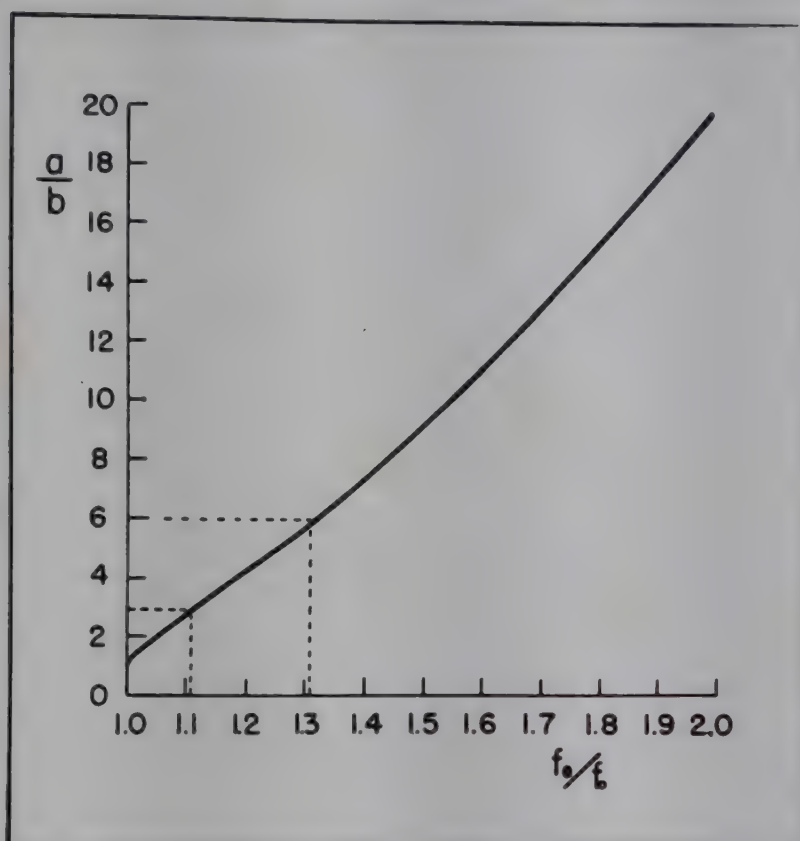


FIG. 18. Graph of Perrin's equation relating the ratio of length to width, a/b , of a molecule, having the assumed shape of a prolate ellipsoid of revolution, to the frictional ratio, f_e/f_0 .

f/f_0 , and the factor, f/f_e , dependent on hydration. Unfortunately it is hard to measure the hydration of a protein unequivocally. Most dissolved proteins probably bind between 0.2 and 0.8 gm. of water per gram of protein.

For illustrative purposes, if we assume the hydration of thyroglobulin to be between these limits and if, further, its molecules approach the shape of prolate ellipsoids of revolution, it is possible to estimate limits for its ratio of molecular length and width as follows.

From the Kraemer relation, the factor, f/f_e , due to hydration is (a) for the lower limit of hydration ($r=0.2$),

$$f/f_e = \left[\frac{0.2 \times 1.00 + 0.720}{0.720} \right]^{1/3} = 1.01,$$

or (b) for the upper limit of hydration ($r=0.8$),

$$f/f_e = \left[\frac{0.8 \times 1.00 + 0.720}{0.720} \right]^{1/3} = 1.04.$$

These limits and the molar frictional ratio, $f/f_0 = 1.42$, previously

TABLE VII. PHYSICAL AND MOLECULAR

	V ₂₀	S ₂₀	D ₂₀	M _{SD}	M _e	M ₅₇
1. σ -Proteose component, milk, cow		0.96 180				
2. Salmin						
3. Soybean seed protein, analytical unit						
4. Nucleohistone, thymus, calf, component (60%)		0.9 181		10,000 181		
5. Insulin, pancreas, dissociation product				12,000 240		
6. Ribonuclease	0.709 ^a 10	1.85 ^a 10	13.6 ^a 10 6.2 ^s 5.3 ^s 6.6 ^s 184	12,700 10	13,000 10	
7. Lysozyme, egg white, hen	(0.70– 0.75) 101 0.75 165	1.9 101 1.8 176	11.2 101 8.6 165	14,000– 17,000 101		18,000 176
8. Cytochrome C	0.707 5	1.9 6	10.1 2	15,600 7		
9. Myoglobin	0.741 8	2.04 2	11.3 2	16,900 7	17,500 2	
10. Myoglobin, heart, horse						
11. Protein, <i>Bacillus phlei</i>	0.748 9	1.8 9	10.2 9	17,000 9		
12. Hemocyanin, <i>Lampetra</i>			10.65 112			
13. Erythrocrucorin, <i>Lampetra</i>	0.751 1	1.87 1	10.7 2	17,100 3	19,000 1	
14. Lactalbumin, milk, cow	(0.751)	1.9 4	10.6 2	17,400 3		
15. Adrenocorticotrophic hormone, sheep		2.1 78	10.5 78	20,000 78		
16. Adrenocorticotrophic hormone, hog		2.04 2.11 79		20,000 78		
17. Phosvitin, egg yolk, hen						
18. Trypsin inhibitor, soybean			8.0 9.1 127			
19. α -Globulin, barley	0.72 170	2.49 170	8.4 170	26,000 170		
20. Toxin, scarlet fever	(0.736)	2.7 67	9.5 67	27,000 67		
21. Hordein, barley	(0.729)	2.0 13	6.5 13	27,500 13		
22. Gliadin, † wheat	0.722 ^b	2.1 11 2.00 139	6.72 12	27,500 ^b 12 28,300 139	27,000 11	
23. Trypsin inhibitor, ovomucoid, egg white, hen						
24. Carbonic anhydrase, red cells, ox	(0.749)	2.8 190 3.80 ^a 189	9.0 190	30,000 190		

CONSTANTS OF PROTEINS

M _{Dη}	M _o	M _n	M _f	M _{el}	M _x	M _i	f/f _o
		8,000 179					
		8,800 202					
		14,300 181					
		12,100 240					
					13,700 105		1.04
12,800 165	17,500 101	(5,000– 12,400) 71 11,920 165, 71			13,900 109		1.58 165
		13,000 138					1.29
							1.11
		17,300 182					
							1.22
							1.16
							1.16
	18,000– 25,000 200						
	24,000 127, 186						
							1.64
			26,000 160			27,000 ^k 139	1.60
	29,000 187 49,000 188						

TABLE VII. PHYSICAL AND MOLECULAR

	V ₂₀	S ₂₀	D ₂₀	M _{sD}	M _e	M _{s7}
25. Chorionic gonadotropin, mare						
26. Crotoxin, venom, rattlesnake	0.704 25	3.14 25	8.6 25	30,000 25	30,500 25	
27. Oxytocic, pressor hormone	(0.749)	2.61 82 1.2- 1.8 81 1.87 ^{hh} 26	8.5 82 4.4 ^{hh} 26	30,000 82 31,000 26		
28. Erythrocrucorin, <i>Chironomus</i>	(0.751)	2.0 15			31,500 1	
29. Carboxypeptidase	(0.75)		8.68 152			
30. Prolactin		2.65 75, 76	7.5 76	32,000 76		
31. Protein, <i>Bacillus tuberculosis</i>	0.70 9	3.3 9	8.2 9	32,000 9		
32. Erythrocrucorin, <i>Arca</i>	(0.751)	3.46 15			33,500 1	
33. Erythrocrucorin ("hemo- globin"), <i>Gastrophilus</i>	(0.75)	2.5				
34. σ -Protease component, milk, cow		2.75 180				
35. Bence-Jones protein, α	0.749 21	3.7 ^o 21			35,000 21	
36. Hemocuprein, horse, ox, sheep				35,000 191		
37. Pepsin (131)	(0.750)	3.3 17 3.0 135	9.0 2 9.69 130	35,500 18	39,000 19	
38. Globin, horse	(0.749) 114	2.57 114	6.5 114	37,000 114		
39. Bence-Jones protein, β	(0.749)	2.8 6	7.3 2	37,000		
40. Chymotrypsin			7.1 128			
41. Chymotrypsinogen	(0.75)	3.1 126	7.9 126	38,000 126		
42. β -Lactoglobulin, goat		3.0 192				
43. β -Lactoglobulin, cow (131)	0.7514 16	3.12 16 3.2 192	7.30 112 7.3- 7.7 184	41,500 16	38,000 16	
44. Insulin (131)	0.749 20	3.5 20 3.34 65 3.55 199	8.2 2	41,000 3 47,000- 48,000 240	35,000 20	
45. Growth hormone, anterior pituitary, ox	0.76 64, 204		7.15 64, 204	49,000 ^y 209		

CONSTANTS OF PROTEINS—*continued*

$M_{D\eta}$	M_o	M_n	M_f	M_{el}	M_x	M_i	f/f_o
		30,000 84					
							1.22
		26,000 82					1.15 82
							1.18 26
							1.63
31,600 152							1.16 152
	26,500 76	25,000 77					
							1.25
							0.99
	34,000	34,600 155					
							0.96
		(2 ×) 18,000 ^t 183					
	37,000 130	34,400 202 34,000 203			40,000 118		1.08
							1.47 114
							1.31
	41,000 128				32,000 119		1.30 128
	36,000 128	36,700 172 36,800 194					1.20 128
	35,050 103 37,300 108	(4 ×) 8,400 202	34,300 104		40,000 125 35,300— 36,500 124 33,000— 35,000 106 35,400— 35,600 193	35,000 107	1.26
	40,000 206	35,100 137, cf. 235			37,600 122		1.13
39,300 64	43,600 204 44,250 64,204	43,600 64					1.31 204

TABLE VII. PHYSICAL AND MOLECULAR

	V ₂₀	S ₂₀	D ₂₀	M _{SD}	M _e	M ₅₇
46. Secalin, rye			4.3 117	40,000 117		
47. Zein, maize	(0.71) 14 (0.73) 198	1.9 14,198	4.0 14,198 3.88 161	40,000 14,198		
48. α-Casein	0.724 ^a 201					
49. Immune pseudoglobulin B, component (6%) colostrum, cow		2- 3 156,227				
50. Metakentrin, sheep		3.6 73				
51. Protein, tuber, potato						
52. Gastric mucoid, group A specific, component, hog		3.6 ^v 6.9 ^v 205	-	40,000 ^w 205		
53. Concanavalin B	0.73 24	3.5 24	7.4 24	42,000 24		
54. Plakalbumin, from ovalbumin, modified by <i>Bacillus subtilis</i> enzyme				43,700 197		
55. Ovalbumin, egg white, hen	0.749 22	3.55 ^d	7.76 12 7.3- 7.7 184 3.96 ^u - 4.14 ^u 196	44,000 12	40,500 23	
56. α-Amylase, pancreas, hog	0.70 207	4.50 207	8.05 207 12.0- 12.5 185	45,000 207 (20,000) 185		
57. Cardiotoxin, venom, cobra				46,200 ^x 208		
58. Fetuin, serum, fetal calf	0.692 61	3.09 61	5.0 61	48,700 61		
59. Fetuin, serum, calf	0.714 61	3.28 61	5.5 61	50,600 61		
60. Amylase, malt	0.69 210	4.52 210	6.53 210	54,000 210		
61. Cucumber seed globulin, analytical unit						
62. Watermelon seed globulin, analytical unit						
63. Fibroin, silk						56,000 15
64. β-Casein	0.740 ^a 201					
65. Pumpkin seed globulin, analytical unit						
66. Squash seed globulin, analytical unit						
67. α-Globulin, mostly pseudo-, serum, human, component		3.6 171				
68. Toxin, Type B, <i>Clostridium botulinum</i>			7.22 226	60,000 ^x 226		

CONSTANTS OF PROTEINS—*continued*

$M_{D\eta}$	M_o	M_n	M_f	M_{el}	M_x	M_i	f_o
						24,000 ^k 198	
		23,000 202				38,000 ^k 198	2.4 14
		(4×)10,000 202					
	40,000 73	40,000 73					
	40,000– 50,000 162						
	70,000; 120,000– 200,000 205						
							1.25
	44,500– 45,000 197						
	34,000– 46,000 131	40,000 137, 195	40,000 160			47,000 141	1.16
		55,000 175					
		55,000 175					
		33,000 202					
		(4×)14,300 202					
		58,000 175					
		58,000 175					

TABLE VII. PHYSICAL AND MOLECULAR

	V ₂₀	n ₂₀	D ₂₀	M _{SD}	M _e	M ₈₇
69. Transaminase, glutamic—aspartic, heart, hog				60,000 232		
70. Chorionic gonadotropin, human	0.76 169	4.3 83, 169	4.4 169	60,000– 80,000 83 100,000 169		
71. Hemoglobin or methemoglobin, horse 131	0.749 27	4.41 6 4.63 135	6.3 28	68,000 63,000 131	68,000 ^e 27	
72. Albumin, serum, fetal calf	(0.736)	4.79 153	6.49 153	67,800 153		
73. Albumin, serum, bovine			6.0 158 6.1 184			
74. Albumin, serum, horse (131)	0.748 29	4.46 ^l 30	6.1 30 6.11 113 6.1– 6.5 129	70,000 ^l 30	68,000 29	
75. Albumin, serum, human	0.733 62 0.736 153	4.6 62 4.67 153	6.1 62 5.93 153	69,000 62 72,300 153		
76. Albumin, serum, horse, mercury derivative		4.6 211				
77. Toxin, <i>Corynebacterium diphtheriae</i>	0.736 32	4.6 32	6.0 32	74,000 32		
78. Toxoid, <i>Corynebacterium diphtheriae</i>		4.6 216				
79. Peptomyosin, muscle, horse, peptic digest						
80. Hemoglobin or carboxyhemoglobin, human	(0.749)	4.46 23 5.3 215	6.9 12 6.7 215	63,000 12 76,000 215		
81. Ricin, castor bean	(0.75) 217			77,000– 85,000 217		
82. Myogen preparation, muscle, rabbit				80,000– 100,000 87		
83. Yellow enzyme	0.731 31	5.76 31	6.3 31	82,000 31	78,000 31	
84. β-Globulin, iron-binding, serum, hog, siderophilin	0.725 219	5.80 219	5.82 219	88,000 219		
85. Tobacco mosaic virus, dissociation component		5.0 254				
86. Metakentrin, hog	(0.749)	5.39 71 6.65 72	5.9 71, 72	90,000 71, 72		
87. β ₁ -Globulin, serum, human	0.725 62	5.5 62		90,000 62		
88. Lipoxidase, soybean				90,000– 100,000 220		
89. Antitoxin, anti-diphtheria	0.749 33, 34	5.5 33, 34	5.76 33, 34	90,500 33, 34		
90. Peptomyosin, muscle, ox, peptic digest						

CONSTANTS OF PROTEINS—*continued*

$M_{D\eta}$	M_o	M_R	M_f	M_{el}	M_x	M_i	f/f_o
		66,700 131, 136 137			66,700 213 69,000 119		1.24
							1.21 153
	69,000 151	(4×)18,400 202, cf. 235					
69,000— 73,000 129	71,700 108 73,000 131	73,000 137					1.27 29, 30 1.21— 1.25 129
	69,000 62, 151						1.28 62 1.30 153
							1.22 32
	75,000 214						
	67,000 116				(See 212)		1.16
							1.2 217
	81,000 87						
							1.17
	93,000 62						1.37 62
							1.23
	91,000 214						

TABLE VII. PHYSICAL AND MOLECULAR

	V_{20}	S_{20}	D_{20}	M_{SD}	M_c	M_{av}
91. Albumin, serum, horse, partly denatured	(0.75) 221	4.4 221	4.5 221	95,000 221		
92. Concanavalin A	0.73 24	6.0 24	5.6 24	96,000 24		
93. Hexokinase, <i>Saccharomyces cerevisiae</i>	0.740 144	3.1 144	2.9 144	96,600 144		
94. Antitoxic (antidiphtheria) pseudoglobulin, pepsin-treated	0.745 32	5.7 32	5.8 32	98,000 32		
95. Dehydrogenase, D-glyceraldehyde-3-phosphate-, muscle, rabbit						
96. Nucleohistone-, thymus, calf, component (40%)	0.729 181	8.7 181		100,000 181		
97. β -Globulin, barley		6.21 170		100,000 170		
98. Tyrosinase, <i>Psalliota campestris</i>	(0.75) 222	6.4 222	6.1 222	100,000 222		
99. Tobacco mosaic virus, dissociation component		8.7 254				
100. Gluten, wheat		2.54- 2.60 146	4.32- 4.34 146		119,000- 129,000 146	
101. Oxidase, L-amino-acid-, kidney, rat, component		5.0 234	4.0 ⁱⁱ 234	120,000 234		
102. Copper-containing protein, serum, human						
103. Phycocyan, <i>Ceramium</i> , dissociation component	(0.746)	6.2 37	4.58 2	130,000 3	146,000 37	
104. Protein, 21-day chick embryo	(0.75)	3.6 236	2.5 236	140,000 236		
105. Aldolase, muscle, rabbit			4.63 225			
106. Globulin X, muscle, rabbit				140,000- 180,000 87		
107. β -(Pseudo) globulin, serum	0.730 171	6.5 171	4.1 171	142,000 171		
108. Albumin, serum, mercury derivative, dimeric		6.5 211				
109. Myogen A, muscle, rabbit	0.735 38	7.86 38	4.78 38	150,000 38		
110. Complement, serum, guinea pig		6.4 69				
111. α -Globulin, mostly pseudo-, serum, component		6.8 171				
112. Complement, serum		6.9 68				
113. Toxin, tetanus, dimeric		7.0 229				
114. β_1 -Globulin, serum, human		7.0 62		150,000 62		
115. γ_1 ($=\beta_2$)-Globulin, serum, human, component		7.0 140				
116. Glucose oxidase, notatin, or penicillin B		8.27 ^{bb} 238	5.13 238	152,000 238		
117. T-Globulin, serum, bovine, component (88%)		7. 150	3.60 ⁱⁱ 149			
118. γ -Globulin A, serum, bovine, component (90%)		7. 150				

TABLE VII. PHYSICAL AND MOLECULAR

	V ₂₀	S ₂₀	D ₂₀	M _{sD}	M _c	M ₈₇
119. γ -Globulin B, serum, bovine, component (92%)		7. 150	3.53 ⁱⁱ 149			
120. Immune pseudoglobulin B, colostrum, cow, component (88%)	(0.75) 150	7. 150, 227 228	3.86 ⁱⁱ 149			
121. Immune euglobulin B, colostrum, cow, component (85%)	(0.75) 150	7. 150, 227	3.34 ⁱⁱ 149			
122. T-Globulin, antitoxic (antitetanus), serum, horse			3.3- 3.5 230			
123. γ -Globulin, serum	0.718 153 (0.745)	7.12 153 7.1 36	4.08 149 4.0 153 3.84 36 2.0 ^s 184	153,000 153 176,000 36		
124. γ -Globulin, serum, component	0.739 62	7.2 62				156,000 62
125. γ_2 (= γ)-Globulin, serum, component		7. 140				
126. Myogen B, muscle				155,000 85		
127. Antipneumococcus globulin, serum, rabbit	(0.745)	6.5 ⁱ 36	3.9 ⁱ 36	158,000 ⁱ 36		
128. Gastric mucoid, group A specific, component, hog		6.9 ^v 205				
129. Immune globulin, colostrum, cow		6.0 227	3.6 149	160,000- 190,000 149		
130. Immune globulin A, colostrum, cow			3.50 149			
131. Immune lactoglobulin (pseudoglobulin), cow			3.48 3.55 150 1.9 ^s 184			
132. γ -Globulin, barley	0.72 170	8.30 170	4.4 170	166,000 170	160,000- 173,000 170	
133. Globulins, total, ^e serum, horse	0.745 29	7.1 35	4.05 2	167,000 3	150,000 19	
134. Anticomplementary pseudoglobulin, serum	0.732 153	7.14 153	3.80 153	170,000 153		
135. Pseudoglobulin fraction GII, serum			4.2 129			
136. Pseudoglobulin fraction GI, serum			4.0 129			
137. γ -Globulin, ^h serum, human	(0.745)	7.1 36	3.84 36	176,000 36		
138. Transaminase, alanine-glutamic acid, heart, hog				180,000 ^{aa} 232		
139. Globulin fraction PII δ_1 , serum, ox	(0.732) 153	7.43 153	3.74 153	180,000 153		
140. Globulin fraction g ₁ δ_1 , serum, ox	(0.732) 153	7.65 153	3.81 153	182,000 153		
141. Immune lactoglobulin fraction, main component (76 to 92%) milk, cow	(0.75) 150	7. 150	3.9 150	180,000 150		
142. Immune lactoglobulin, euglobulin fraction, cow			3.10 3.38 150			

CONSTANTS OF PROTEINS—*continued*

$M_D\eta$	M_o	M_a	M_f	M_{el}	M_x	M_i	f/f_o
	153,000 108						1.51 153 1.49
	155,000– 200,000 ⁱⁱ 62						1.38 62
							1.52
	120,000– 200,000 205						
							1.44
							1.53 153
170,000 129							1.39 129
174,000 129							1.42 129
							1.49
							1.52 153
							1.49 153

TABLE VII. PHYSICAL AND MOLECULAR

	V ₂₀	S ₂₀	D ₂₀	M _{nD}	M _e	M _{n7}
143. Antitoxic (antidiphtheria) pseudoglobulin, serum	(0.745) 32	7.2 32	3.9 32	184,000 32		
144. Antitoxic (antitetanus) γ -globulin, serum, horse			3.9 178, 230			
145. Globulin fraction— $g_1\delta_1$, serum, ox	(0.732) 153	7.61 153	3.58 153	192,000 153		
146. Antipneumococcal globulin, serum, human	(0.745)	7.4 36	3.60 36	195,000 36		
147. α_1 -Globulin, serum, human	0.841 62	5.0 62				200,000 62
148. Pseudoglobulin fraction GIII, serum			3.9 129			
149. γ -Globulin, wheat embryo	0.72 166	8.7 166	3.6 166	213,000 166		
150. Tobacco mosaic virus, dissociation component		8.7 254				
151. Starch phosphorylase, tuber, potato		9.2 168				
152. γ_1 ($=\beta_2$)-Globulin, component, serum, human		9 140				
153. γ_2 ($=\gamma$)-Globulin, component, serum, human		9 140				
154. Protein, 12 day chick embryo	(0.75)	4.0 236	1.6 236	240,000 236		
155. Catalase	0.73 43	11.3 43	4.1 43 4.5 ^a 134	250,000 43		
156. Phycocyan, main component, <i>Ceramium</i>	(0.746)	11.4 37	4.05 28	270,000 7	275,000 37	
157. "Polyhedral" cytoplasmic inclusion, <i>Porthretia dispar</i>	0.736 243	12.57 243	4.18 243	276,000 243		
158. Phycoerythrin, <i>Ceramium</i>	0.746 22	12.0 37	4.00 28	290,000 18	290,000 37	
159. Excelsin	0.743 40	13.3 ^e 40	4.26 2	295,000 18		
160. δ -Globulin, barley		12.0 170		300,000 ^y 170		
161. Peptophan						
162. γ -Globulin, component, serum, human		10 62				300,000 62
163. α_2 -Globulin, serum, human	0.639 62	9 62				300,000 62
164. T-Globulin, component (12%), serum, ox		10 150				
165. γ -Globulin, fraction A, component (10%), serum, ox		10 150				
166. γ -Globulin, fraction B, component (8%), serum, ox		10 150				
167. Immune pseudoglobulin B, colostrum, cow, component (6%)		10 150, 227				
168. Immune euglobulin B, colostrum, cow, component (5%)		10 150, 227				
169. Casein (ogen), " δ ", milk, cow		10.4 ^j 154				
170. Edestin 131	0.744 39	12.8 39	3.17 115	310,000 ^o 115		

CONSTANTS OF PROTEINS—continued

TABLE VII. PHYSICAL AND MOLECULAR

	V ₂₀	S ₂₀	D ₂₀	M _{sD}	M _e	M ₈₇
171. Globulin, seed, tobacco		12.7 102				
172. Amandin	0.746 40	12.5 ^c 40	3.45 115	330,000 ^P 41	330,000 42	
173. "Polyhedral" cytoplasmic inclusion, <i>Lymantria monacha</i>	0.736 243	12.78 243	3.50 243	336,000 243		
174. Phosphorylase, muscle, rabbit	(0.74)	13.7 86	3.2- 3.8 86	340,000- 400,000 86		
175. Globulin, serum, <i>Lampetra</i>	(0.745)	12.0 44	3.2 44	360,000 44		
176. "Polyhedral" cytoplasmic inclusion, <i>Bombyx mori</i>	0.736 243	12.85 243	3.12 243	378,000 243		
177. Hemocyanin, dissociation component, <i>Busycon</i>	(0.738)	13.5 45	3.29 45	380,000 45		
178. Globulin, seed, beet		13.9 231				
179. Urease, light-inactivated		15.0 248				
180. Erythrocrucorin, <i>Daphnia</i>		16.3 1				
181. Hemocyanin, <i>Pandalus</i>	(0.740)	17.4 45			400,000 45	
182. Fibrinogen, plasma, human		9 62	dd 244			400,000 62
183. Hemocyanin, <i>Palinurus</i>	(0.740)	16.4 45	3.4 2	450,000 18	450,000 45	
184. Hemocyanin, <i>Eledone</i> , dissociation component	(0.740)	10.6 45	2.16 2	460,000 45		
185. Apoferritin, horse or human	(0.747) 110	17.6 110	3.61 110	467,000 110		
186. Oxidase, L-amino acid, kidney, rat, component		13.5 234	3.0 234	480,000 234		
187. Urease	0.73 46	18.6 46	3.46 46	480,000 46		
188. Urease, component (80%)		18.2 248				
189. Hemocyanin, <i>Helix pomatia</i> , dissociation component	(0.738)	12.1 45	2.23 45	500,000 45		
190. Isohemagglutinin, human	(0.73) 153	19.8 153		500,000 153		
191. Thyroglobulin, hog	0.72 47	19.2 47	2.65 2	630,000 3	650,000 47	
192. α-Globulin, mostly <i>pseudo</i> -, serum, human, component		17 171				
193. γ ₁ (=β ₂)-Globulin, serum, human, component (30%)		18 140				
194. Oxidase, L-amino acid, kidney, rat, component		18 234				
195. Hemocyanin, <i>Helix pomatia</i> , dissociation component	(0.738)	16.0 45	2.06 2	720,000 45	800,000 45	
196. Hemocyanin, <i>Homarus</i>	(0.740)	22.6 45	2.78 2, 112	760,000	800,000 45	
197. Hemocyanin, <i>Helix nemoralis</i> , dissociation component	(0.738)	16.6 45	1.92 2	800,000 45		
198. Immune euglobulin B, component (10%) colostrum, cow	(0.75) 150	20 150, 227				
199. Whey component (10%), cow		20 227				

CONSTANTS OF PROTEINS—*continued*

M_{D7}	M_o	M_a	M_f	M_{el}	M_x	M_i	f/f_o
		56,000 175			325,000 123		
	206,000 121						1.28
							1.2- 1.4
							1.41
							1.35
							1.07
	580,000 62						1.98 62
							1.23
							1.93
					460,000 111		1.14 110
							1.19
							1.81
							1.43
							1.74
							1.27
							1.80

TABLE VII. PHYSICAL AND MOLECULAR

	V ₂₀	S ₂₀	D ₂₀	M _{sD}	M _e	M ₈₇
200. Hemocyanin, <i>Nephrops</i>	(0.740)	24.5 45	2.79 2	820,000 3		
201. Toxin, type A, <i>Clostridium botulinum</i>	0.755 143 0.736 246	17.3 100, 246	2.14 100 2.10 143 1.87 246	900,000 100, 246 246		
202. Globulin, antipneumococcus, serum, horse	0.715 36, 48	19.3 36, See 110	1.80 36, See 110	910,000 36		
203. Globulin, antipneumococcus, serum, ox	(0.715)	18.1 36, 48	1.69 36, 48	910,000 36, 48		
204. Globulin, antipneumococcus, serum, hog	(0.715)	18.0 36, 48	1.64 36, 48	930,000 36, 48		
205. Urease, component (20%)		27.5 248				
206. Virus, poliomyelitis, Lansing		76.2- 90.9 145				
207. Euglobulin, pathological serum, human	0.753 153	19.1- 19.5 153	1.5 153	1,200,000 153		
208. Hemocyanin, <i>Calocaris</i>	(0.740)	34 15			1,330,000 15	
209. Myosin		7.2 241	0.5 241	1,500,000 241		
210. Nucleoprotein, liver, rabbit		26.3 249	0.78- 1.78 249	1,600,000- 2,300,000 249		
211. Erythrocrucorin, <i>Planorbis</i>	0.745 1	33.7 1, 15	1.96 12	1,630,000 12	1,540,000 19	
212. Virus, alfalfa mosaic		74 93, 94		2,000,000 ^y 93, 94		
213. Nucleohistone, thymus, calf	0.658 50	31.0 50	0.93 50	2,300,000 50	2,000,000 50	
214. Virus, yellow fever	0.87 250	27- 45 250		2,500,000 ^y 250		
215. X-protein (β-globulin), serum, human	0.97 ^{cc} 60	5.9 60	1.7 60	2,600,000 ^{cc} 60		
216. Virus, tobacco necrosis		49 164				
217. Hemocyanin, <i>Eledone</i>	(0.740)	49.1 45	1.64 2	2,800,000 18		
218. Hemocyanin, <i>Octopus</i>	0.740 49	49.3 45	1.65 41	2,800,000 41		
219. Chlorocruorin, <i>Spirographis</i>		55.2 19				
220. Virus, tobacco ringspot		115 91			3,000,000 ^y 91	
221. Erythrocrucorin, <i>Arenicola</i>	(0.740)	57.4 51		3,000,000 19		
222. Erythrocrucorin, <i>Lumbricus</i>	0.740 51	60.9 51	1.81 2	3,150,000 3	2,950,000 19	
223. Hemocyanin, <i>Rossia</i>	(0.740)	56.2	1.58 2	3,300,000 18		
224. Hemocyanin, <i>Limulus</i>		60.5 156		3,400,000 ^y 156		
225. Virus, turnip yellow mosaic						

CONSTANTS OF PROTEINS—*continued*

$M_{D\eta}$	M_o	M_a	M_f	M_{el}	M_x	M_i	f/f_o
							1.23
1,130,000 143		45,000 245					1.76 100,246 1.45 143
							1.86 See 110
							1.98
							2.02
				900,000– 21,000,000 ^r 145			
							1.22
	1,000,000 87						
							2.6– 3.3 249
							1.39
							2.5
	370,000 239						
				See 255			
							1.39
							1.38
							1.25
							1.21
							1.36
				3,500,000 256			

TABLE VII. PHYSICAL AND MOLECULAR

	V ₁₀	S ₂₀	D ₂₀	M _{SD}	M _e	M ₈₇
226. Virus, tobacco mosaic, dissociation product		97 254				
227. Virus, tobacco necrosis 1		112 91, 95		6,000,000 ^y 91, 95		
228. Hemocyanin, <i>Busycon</i> , main component	(0.738)	101.7 45	1.38 45	6,800,000 45		
229. Virus, bean mosaic						
230. Virus, tomato bushy stunt 131	0.739 63	146 53 132 54, 55	1.15 58	10,600,000 58	7,600,000 53	
231. Hemocyanin, <i>Paludina vivipara</i>	0.738 63	102.5 63	1.09 63	8,700,000 63		
232. Hemocyanin, <i>Helix pomatia</i> , main component	0.738 52	103 63	1.07 63	8,910,000 63	6,700,000 45	
233. Hemocyanin, <i>Busycon</i> , aggregation component	(0.738)	130 45	1.17 45	10,000,000 45		
234. Virus, tobacco mosaic, dissociation component		125 254		14,800,000 ^y 254 24,000,000 ^y 254 27,100,000 ^y 254 34,000,000 ^y 254		
235. Virus, potato ringspot		130 91		26,000,000 91		
236. Myosin	(0.75)	12.0 242	0.30 242	39,000,000 242		
237. Virus, tobacco mosaic. a. ordinary strain, from tobacco	0.727 88	174 89 185 88 170 177 188 ^{ff} 252	0.3 89 0.53 88	59,000,000 89 31,600,000 88		42,600,000 173 33,200,000 88
b. ordinary strain, from phlox		176 177				
c. strain TM-58, from tobacco	0.734 251	198 251	0.46 251	40,700,000 251		
d. ribgrass strain, Holmes', from tobacco		187 ^{ff} 252				
from tobacco		186 177				
from phlox		176 177				
e. green aucuba, from tobacco		192 ^{ff} 252				
f. yellow aucuba, from tobacco		191 ^{ff} 252				
g. masked, Holmes' from tobacco		180 ^{ff} 252				
h. strain J 14 D1, from tobacco		185 ^{ff} 252				
238. Virus, cucumber 3		187 91, 92 186 ^{ff} 252		40,000,000 ^y 91, 92		
239. Virus, cucumber 4		187 91, 92 181 ^{ff} 252		40,000,000 ^y 91, 92		
240. Virus, rabbit papilloma	0.756 56 0.761 148	280 56 297 148	0.51 56 (0.72) 148	47,100,000 56		
241. Allantoic fluid component, chick		220 258				

CONSTANTS OF PROTEINS—*continued*

M _{D7}	M _o	M _a	M _f	M _{cl}	M _x	M _i	f/f _o
				See 257			
				7,000,000 See 133			
				7,000,000 See 133	12,800,000 120	9,000,000 ⁿ 132	1.09 1.27
							1.43 63
							1.24
						24,000,000 ^m 163	
40,000,000 88				41,000,000 167, See 252, 253	39,800,000 54, 167 40,000,000 88		3.12 89
							2.03 251
							1.49

TABLE VII. PHYSICAL AND MOLECULAR

	V_{20}	S_{20}	D_{20}	M_{SD}	M_e	M_{87}
242. Virus, equine encephalitis		253 99				
243. Thromboplastic protein, lung	0.87 66	330 66	0.38 66	167,000,000 66		
244. Hemagglutinating component, noninfective, allantoic fluid, chick, after influenza infection, Type A, Pr 8		380 258				
245. Virus, chicken sarcoma		550 97, 98				
246. Actomyosin						
247. Virus, influenza, Type A, a. W S		674 700 ^{gg} 59				
b. PR8		700 59 660 258				
c. F-12		700 ^{gg} 59				
d. Weiss		700 ^{gg} 59				
e. Swine	0.79 59	700 ^{gg} 59				
f. Type B, Lee	0.79 59 0.863 259	800 89 802 259 See 258				
248. T ₁ -Bacteriophage of <i>Escherichia coli</i> associated form	0.665- 0.669 147	700 142, 145 1,000 142, 145				
249. Allantoic fluid component (minor), chick		1,330 258				
250. Virus, polyhedral, <i>Bombyx mori</i>	0.770 243	1,871 243	2.18 243	1,000,000,000- 2,000,000,000 243		
251. Virus, vaccinia		4,910 96				

LEGEND AND NOTES FOR TABLE VII

V_{20} = partial specific volume of the protein, cm.³/g. (The values in brackets are assumed values.)

S_{20} = sedimentation constant in Svedberg units referred to water at 20°, seconds ($\times 10^{13}$).

D_{20} = diffusion constant referred to water at 20°, cm.²/sec. ($\times 10^7$).

M_{SD} = molecular weight computed from sedimentation velocity and diffusion measurements.

M_e = molecular weight computed from sedimentation equilibrium measurements.

M_{87} = molecular weight computed from the sedimentation constant and intrinsic viscosity.

$M_{D\eta}$ = molecular weight computed from the diffusion constant and intrinsic viscosity.

M_o = molecular weight computed from osmotic pressure.

M_x = molecular weight computed from x-ray analysis.

M_n = molecular weight computed from a minimum molecular weight indicated by chemical analysis.

M_f = molecular weight computed from surface film measurements.

M_{el} = molecular weight computed from electron microscope measurements.

M_i = molecular weight computed from light scattering measurements.

f/f_o = molar frictional ratio (ratio of experimentally determined molar frictional constant to molar frictional constant calculated for an unsolvated spherical particle of the same mass).

The reported f/f_o values are probably significant only within ± 0.03 and are sometimes even less accurate than this.

† McCalla and Gralén (57) have studied wheat gluten in sodium salicylate solutions. The preparations were polydisperse, the weight-average molecular weight of the most soluble fraction was 44,000, but the minimum molecular weight of particles in this fraction may have been as low as 35,000.

^a Values at 25°.

^b The specific volume of gliadin varies strongly with the protein concentration, according to Krejci and Svedberg (11). For the calculation of M_n Lamm and Polson (12) used $V = 0.722$. Arrhenius (139) used $V = 0.745$, $D_{20} = 6.78$, and $M_n = 28,300$ as an average value for three different gliadin fractions.

^c Recalculated to water basis.

^d Mean value obtained by a number of different observers in Upsala.

^e Mean of two determinations on CO-hemoglobin and two on methemoglobin.

^f Mean values for the A and B fractions prepared by Kekwick (30).

^g Total serum globulin obtained by half saturation with ammonium sulfate.

^h Electrophoretically prepared γ -globulin from normal human serum.

ⁱ Mean of four determinations on four different specimens.

CONSTANTS OF PROTEINS—continued

$M_{D\eta}$	M_o	M_n	M_f	M_{el}	M_x	M_i	f/f_o
							1.41 66
						300,000,000 ^m 163	
				200,000,000 See 59, 258		322,000,000 ⁿ 132	
				See 243			

^j The sedimentation constant of the principal (δ) casein (ogen) component is strongly concentration dependent. Molecular weights of Van Slyke-Baker preparations are reported as ranging from 75,000 to 100,000; a fraction of the still more heterogeneous Hammarsten preparation had a molecular weight of 375,000. The particles of calcium caseinate in milk are many times larger: 20 to 140 $m\mu$ in diameter (154).

^k Determined from dielectric properties.

^l Estimated from the molecular volume computed by the authors (157) from measurement of flow birefringence (density; 1.425).

^m From data on viscosity, flow birefringence, and light scattering.

ⁿ By light absorption.

^o Using $s = 3.93$ (2).

^p Using $s = 3.62$ (41).

^q The molecular weight of catalase, as spheres, computed from the diffusion constant is 350,000 to 375,000 (134).

^r Estimated from the diameter cited: $25 \pm 12 m\mu$ (145), taking the density to be 1.33.

^s Referred to 0°, 0.1 N NaCl.

^t Serum protein perhaps identical with hemocuprein, from copper content.

^u Referred to water at 0°. This confirmed Polson's values for 20°.

^v As measured, 0.5% NaCl at 22.5°.

^w Estimated minimum molecular weight from sedimentation constant alone.

^x Estimated from the diffusion constant alone.

^y Estimated from sedimentation data.

^z For electrophoretically single catecholase fraction, from copper content.

^{aa} Estimated from data not cited.

^{bb} At 0.36 gm. per 100 ml. There is an appreciable concentration effect.

^{cc} Hydrated.

^{dd} Edsall, *et al.* report a rotary diffusion constant, $\theta_{20, w} = 35,000$ second,⁻¹ and molecular dimensions, from flow birefringence.

^{ee} Computed from the reported iron content.

^{ff} As observed in 0.1 M phosphate at a virus concentration of 2.0 to 3.3 mg./ml.

^{gg} Sedimentation constants corrected for the viscosity of the solution, containing variable amounts of a viscous impurity.

^{hh} The sedimentation constant is as measured at 6.3°; the diffusion constant, as measured at 0.3°.

ⁱⁱ Value(s) determined for the unfractionated preparation.

^{jj} "Leakage."

found for this protein permit estimation of limiting values for the factor, f_e/f_0 , accounting for the effect of molecular shape. (a) For the lower limit of hydration ($r=0.2$)

$$f_e/f_0 = \frac{f/f_0}{f/f_e} = \frac{1.42}{1.01} = 1.41;$$

for the upper limit of hydration ($r=0.8$)

$$f_e/f_0 = \frac{f/f_0}{f/f_e} = \frac{1.42}{1.04} = 1.37.$$

Referring to Figure 18, the graph of the Perrin equation, we find that the thyroglobulin molecule is from seven to eight times as long as it is wide, under the stated assumptions.

V. CONCLUSION

Table VII gives molecular constants for many proteins, extending the lists of Svedberg and Pedersen (90, p. 406) and Cohn and Edsall (19, pp. 428–31). Though molecular weights estimated in different ways often agree very well, this is not always to be expected with a new, uncharacterized material. Different methods depend upon a variety of properties not related in the same way to molecular weight. Osmotic pressure, for instance, estimates the number of independent kinetic units per unit volume. If a variety of sizes of molecules is present, this method gives a molecular weight that is a number-average.

On the other hand, sedimentation depends more directly on the weight of the sedimenting unit. Molecular weights based on sedimentation may, depending upon the method used in analyzing the data, give an average value approximating a weight-average. Since the number-average, weight-average and other averages are the same only for a solute of uniform molecular weight, coincidence of molecular weights estimated by several methods is an important necessary criterion of homogeneity. From theoretical considerations it is also possible to combine data from the various methods to get indexes of the degree of inhomogeneity of a material. The student is referred to *The Ultracentrifuge* (90, pp. 325–53) and to Jullander (45) for a discussion of the relations of various averages to the dispersity of the solute.

It is evidently good practice to measure molecular constants of a protein suspected of being a pure substance by all available methods. It is implied, and some of the evidence is given in Table VII, that proteins in general are remarkably homogeneous. This characteristic is specially striking in comparison with other macromolecular systems such as starches, celluloses, or synthetic high polymers.

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Chapter VII

AMPHOTERIC PROPERTIES OF AMINO ACIDS AND PROTEINS

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I. INTRODUCTION

MANY of the important physical-chemical and biological properties of the amino acids and proteins stem from their amphoteric nature. This permits them to exist, according to the pH of the solution, either as electrically neutral molecules or as positively or negatively charged ions.

The characteristic amphoteric properties have proved useful in determining the degree of purity of amino acids (16), in establishing the structure of peptides (glutathione 29, 57), as the basis of a method of analysis for certain amino acids in proteins (31), and to indicate the presence of certain amino acids with specific ionizable groups in proteins. The isoelectric point provides a useful constant for the characterization of proteins, peptides and certain amino acids. Biologically, the amphoteric nature of proteins is important for the buffering of body fluids and tissues and for the formation of important conjugated proteins (nucleoproteins, nucleotide-proteins, iron porphyrin-proteins, metallo-proteins).

Hitchcock (62) points out that although the first amino acids were discovered long before the formulation of the ionic theory, their amphoteric nature was immediately recognized. Compounds of amino acids with acids and with bases were soon isolated, and their formation was accounted for by the basic properties of the amino groups and the acidic properties of the carboxyl groups.

Strecker (67) explained the neutral character of glycine solutions by assuming that the acid and basic groups of the molecule interacted, forming an internal salt. This idea was more fully developed by Bredig (6) and by Bjerrum (4) into the concept of the dipolar ion (Zwitterion); an ion that carries both a positive and a negative charge, located on different groups of the same molecule.

The mathematical development of the theory of amphoteric dissociation, as that of electrolytic dissociation in general, has reached a state of great complexity. It would require far too much space and it is needless for the present purpose to give a complete presentation of all the details of the mathematical aspects of amphoteric dissociation. Consequently, the mathematical derivation of only such phases of the subject as are essential for the desired purpose will be presented and in many instances these will be given in an approximate and simplified form. For a more complete and more rigidly exact presentation the reader is referred to Cohn and Edsall (12) or to the original papers cited in the references.

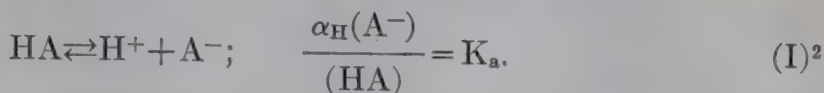
II. ELECTROLYTIC DISSOCIATION

The key to the understanding of the properties of electrolytes was discovered by Arrhenius (1). He showed that their high electrical conductivity and other properties could be explained by the assumption that in aqueous solution electrolytes are dissociated into electrically charged positive and negative ions. Following this discovery, it soon became apparent that these substances,

in the main, grouped themselves into two classes: strong electrolytes—like HCl, NaOH, and NaCl—which are essentially completely dissociated into ions in solution, and weak electrolytes which are incompletely or only slightly dissociated. To the latter group belong most organic acids and bases and the amino acids and proteins.

An important distinction between the two groups is that the dissociation of weak electrolytes, but not that of strong electrolytes,¹ can be formulated as an equilibrium in terms of the law of mass action, in which the active masses of the dissociated ions and of the undissociated residue can be represented by their molal concentrations.

For example the electrolytic dissociation of a weak mono acid—e.g. acetic acid can be represented by the simple equation



As a measure of the acidity or alkalinity the logarithmic functions given below are employed virtually universally

$$\text{pH} = -\log \alpha_{\text{H}} \quad \text{and} \quad \text{pOH} = -\log \alpha_{\text{OH}}. \quad (\text{II})$$

The dissociation of water can be represented by the equation

$$\alpha_{\text{H}} \cdot \alpha_{\text{OH}} = K_{\text{w}} \quad \text{or} \quad \text{pH} + \text{pOH} = \text{p}K_{\text{w}}.$$

From this it follows that the alkalinity as well as the acidity of a solution can be represented by the pH, making the function pOH of only occasional usefulness.

To take advantage of the logarithmic representation of acidity, equation I is converted into logarithmic terms in the manner shown below:

$$\log \frac{1}{\alpha_{\text{H}}} = \log \frac{1}{K_{\text{a}}} + \log \frac{(\text{A}^-)}{(\text{HA})}$$

¹ Thermodynamic analysis shows that the mass law equation of any electrolyte, strong or weak, is valid if activities are employed instead of concentrations (39).

² In solutions of weak electrolytes and in mixtures of electrolytes, hydrogen and hydroxide ions are determined electrometrically or by some equivalent procedure. Such a measurement yields the activity of the hydrogen ion (α_{H}) or the hydroxyl ion (α_{OH}) rather than concentration. For this reason the activities are used in the mass law equations.

Concentrations of hydrogen and hydroxyl ions are calculated by means of the equations

$$(\text{H}^+) = \frac{\alpha_{\text{H}}}{\gamma_{\text{H}}} \quad \text{and} \quad (\text{OH}^-) = \frac{\alpha_{\text{OH}}}{\gamma_{\text{OH}}}$$

where γ_{H} and γ_{OH} represent the activity coefficients of these two ions respectively. Evaluation of the activity coefficients of these ions in solution containing mixtures of electrolyte is a problem fraught with great difficulties (23).

or

$$\text{pH} = \text{pK}_a + \log \frac{(\text{A}^-)}{(\text{HA})}$$

Making the still further approximation that the anion concentration is equivalent to the concentration of the salt of the weak acid (BA)³ the above equation now takes the form

$$\text{pH} = \text{pK}_a + \log \frac{(\text{BA})}{(\text{HA})} \quad (\text{IIIa})^4$$

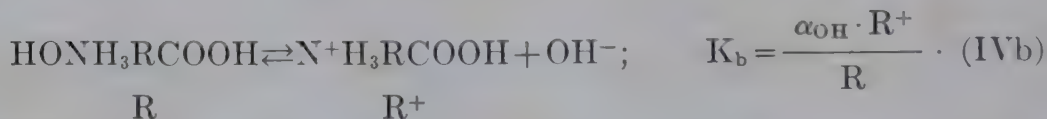
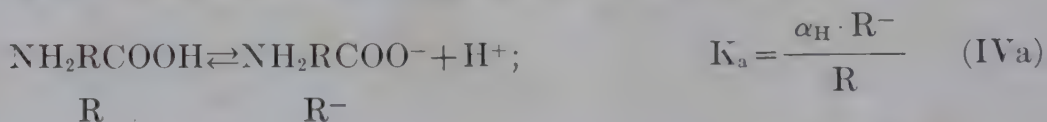
The equivalent equation for the dissociation of a weak base is:

$$\text{pH} = \text{pK}_w - \text{pK}_b + \log \frac{(\text{ROH})}{(\text{RX})} \quad (\text{IIIb})$$

III. FORMULATION OF THE DISSOCIATION OF AMINO ACIDS

1. Classical Formulation

The ionization of an amino acid containing one basic and one acid group was originally represented by the equations:



³ A more exact derivation is to employ activities instead of concentrations in the following manner

$$\text{pH} = \text{pK}_a + \log \frac{\gamma_s(\text{BA})}{\gamma_A(\text{HA})}$$

where γ_s represents the activity coefficient of the salt and γ_A of the free acid. The activity coefficients can be merged into the dissociation constants as follows:

$$\text{pH} = \log \frac{\gamma_s}{\text{K}_a \gamma_A} + \log \frac{(\text{BA})}{(\text{HA})}$$

The term $\log \gamma_s / \text{K}_a \gamma_A$ is combined and written as pK'_a and represents an apparent and approximate dissociation constant. In very dilute solutions the values of the activity coefficients approach unity and the true value of the pK is reached.

⁴ Equations IIIa and IIIb are particularly useful for the representation of dissociation curves and the properties of buffer solutions. By introducing a term, degree of dissociation, defined by the relation

$$\alpha = \frac{(\text{BA})}{(\text{HA}) + (\text{BA})}$$

the above equations can be transformed into

$$\text{pH} = \text{pK}_a + \log \frac{\alpha}{1 - \alpha}$$

and

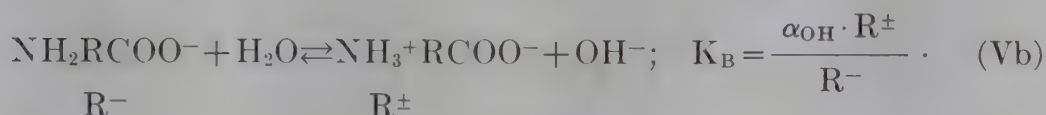
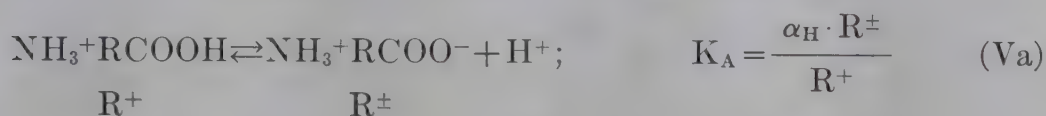
$$\text{pH} = \text{pK}_w - \text{pK}_b + \log \frac{1 - \alpha}{\alpha}$$

The terms R , R^- , and R^+ , without parentheses, are employed to represent concentrations of the different amino acids species.

Values for the dissociation constants of the amino acids so formulated were found to be astonishingly small in comparison to the dissociation constants of the parent fatty acid or amine; namely, of the order of 10^{-9} to 10^{-10} for K_a and 10^{-10} to 10^{-12} for K_b .

2. Dipolar Ion Formulation

A large body of evidence has accumulated which shows that amino acids and proteins are internal salts, *i.e.*, have a dipolar ion structure in solution. This structure leads to the formulation for the dissociation equations of amino acids shown below:



What was formerly thought to be the uncharged amino acid is seen to be an amphoteric ion. It will be noted that the neutralization of an acid solution with base causes the dissociation of the proton of the carboxyl group, and of an alkaline solution with acid the addition of a proton to the amino group. The nature of the acid-base reactions of a dipolar ion can be made clearer by considering the titration of a solution like ammonium acetate.

With acid the reaction is



The titration with H^+ , it will be noted, back-titrates the carboxyl group and thus is a measure of its strength. Similarly, titration with OH^- back-titrates the ammonia and is a measure of its strength.



The same situation prevails when an amino acid is titrated. Since R and R^\pm represent the same substance, the relation between the classical and dipolar ion constants can be obtained by multiplying together equations Va and IVb and equations Vb and IVa which yield

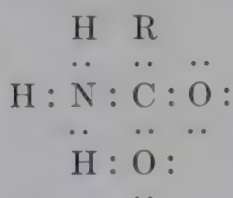
$$K_A \cdot K_b = \alpha_H \cdot \alpha_{OH} = K_w = K_B K_a.$$

Consequently

$$K_A = \frac{K_w}{K_b} \quad \text{and} \quad K_B = \frac{K_w}{K_a} \quad (\text{VI})$$

It is worthwhile at this point to consider the evidence in favor of the dipolar ion structure of amino acids. This is listed below:

- (a) The values of the constants, $K_A = 10^{-2}$ to 10^{-3} and $K_B = 10^{-4}$ to 10^{-5} are in more reasonable agreement with the chemical structure.
- (b) The amino acids are crystalline compounds that decompose before melting or boiling. This behavior is characteristic of the salts but not of the parent fatty acids or amines of the amino acids.
- (c) The high dielectric constants and high dipole moments of the amino acids are in harmony with the dipolar ion structure.
- (d) On the basis of the Lewis octet atom, the dipolar ion structure leads to a more symmetrical molecule



- (e) Direct evidence is derived from the Raman spectra of amino acids. Examples are given in Fig. 1. A spectral line at 1700 cm^{-1} , characteristic of the carboxyl group, does not occur in neutral solutions of amino acids but is found in solutions of their hydrochlorides. The obvious deduction is that the carboxyl group is ionized in neutral solution.
- (f) Less direct evidence is the finding of Harris (24, 25) that the alkali titration curve of amino acids is shifted to a more acid region by formaldehyde. Similarly, formaldehyde influences the titrations of ammonia but not of acetic acid. This leads to the conclusion that the basis of the change in the titration curve with alkali is the effect on the basic strength of the amino group produced by the reaction with formaldehyde.

3. Brönsted Acid Dissociation Formulation

G. N. Lewis (40) has pointed out that, in terms of the electronic theory of chemical structure, an *acid* is an atom or molecule capable

Raman Frequencies of Fatty Acids and Amino Acids.

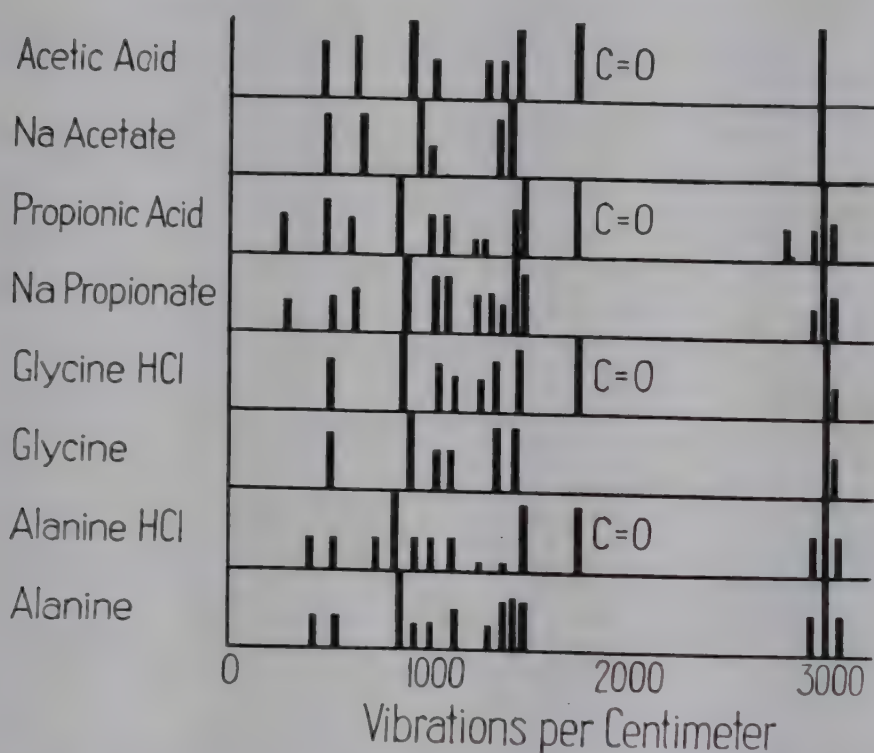
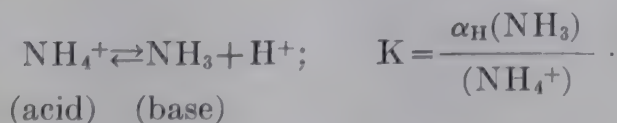


FIG. 1. Raman frequencies of fatty acids and amino acids. The Raman frequencies are plotted in reciprocal wave lengths (cm^{-1}). The height of each line is roughly proportional to its relative intensity. Edsall, J. T.: *J. Chem. Phys.*, 4:1 (1936).

of sharing a pair of electrons and, conversely, a *base* is an atom or molecule that can donate an electron-pair to form a coördinate bond. Neutralization is the formation of a covalent bond between the acid and base.

A special case of the above definition of particular usefulness in non-aqueous or mixed solvents is the formulation of Brönsted (7) that an acid is a substance capable of dissociating off hydrogen ion; a base is a substance capable of uniting with hydrogen ion. A good example is ammonia, whose dissociation can be represented by:



One particularly useful result of the Brönsted system is that it eliminates the need for employing K_w in the dissociation equations.

TABLE I

APPARENT DISSOCIATION CONSTANTS OF AMINO ACIDS, PEPTIDES
AND RELATED SUBSTANCES DETERMINED FROM EMF MEAS-
UREMENTS ON CELLS WITH LIQUID JUNCTION AT 25°
(Compiled by Edsall (12) p. 84)

A. Substances Containing One Amino and One Carboxyl Group			
Substance	pK ₁ '(COOH)	pK ₂ '(NH ₃ ⁺)	pI
1. Amino acids			
Glycine	2.34	9.60	5.97
Alanine	2.34	9.69	6.00
α-Amino- <i>n</i> -butyric acid	2.55	9.60	6.08
Threonine	2.71	9.62	6.16
Valine	2.32	9.62	5.96
α-Amino- <i>n</i> -valeric acid	2.36	9.72	6.04
Leucine	2.36	9.60	5.98
Isoleucine	2.36	9.68	6.02
Norleucine	2.39	9.76	6.08
Serine	2.21	9.15	5.68
Proline	1.99	10.60	6.30
Hydroxyproline	1.92	9.73	5.83
Phenylalanine	1.83	9.13	5.48
Tryptophan	2.38	9.39	5.89
Methionine	2.28	9.21	5.74
Isoserine	2.78	9.27	6.02
Hydroxyvaline	2.61	9.71	6.15
Taurine	1.5 (SO ₃ H)	8.74	5.12
β-Alanine	3.60	10.19	6.90
γ-Amino- <i>n</i> -valeric acid	4.02	10.40	7.21
δ-Amino- <i>n</i> -valeric acid	4.270*	10.766*	7.518
ε-Amino- <i>n</i> -caproic acid	4.43	10.75	7.59
ω-Amino- <i>n</i> -dodecanoic acid	4.648	—	—
2. Amino acid amides			
Glycine amide	—	7.93	—
Glutamine (γ-amide)	2.17	9.13	5.65
Asparagine (β-amide)	2.02	8.80	5.41
β-Hydroxyasparagine	2.12	8.26	5.19
Isoglutamine (α-amide)	3.81	7.88	5.85
Isoasparagine (α-amide)	2.97	8.02	5.50
α-Hydroxyasparagine	2.31	7.17	4.74
3. Peptides			
Glycylglycine	3.06	8.13	5.60
	3.12	8.17	5.65
	3.083*	8.265*	5.674
Glycylalanine	3.15	8.25	5.70
Glycylleucine	3.18	8.29	5.73
Glycylvaline	3.17	8.25	5.71
Alanylalanine	3.17	8.42	5.79
Alanylglycine	3.11	8.18	5.64
α-Aminobutyryl-α-aminobutyric acid	3.04	8.39	5.72
Glycylproline	2.84	8.53	5.69
Alanylproline	3.04	8.38	5.71
Alanyldiglycine	3.21	8.15	5.68
Glycylalanylalanylglycine	3.30	7.93	5.62
Leucyl-octaglycylglycine	(2.27)	(7.84)	(5.03)
Phenylalanylglycine	3.10	7.71	5.41
Triglycine	3.26	7.91	5.59
Tetraglycine	3.05	7.75	5.40
Pentaglycine	3.05	7.60	5.32
Hexaglycine	3.05	7.60	5.32
4. Amino acids and peptides containing methylated amino groups			
Substance	pK ₁ '(COOH)	pK ₁ '(Amino)	pI
Sarcosine	2.23	10.01	6.12
Sarcosylglycine	3.10	8.51	5.80
Glycylsarcosine	2.83	8.54	5.68
Sarcosylsarcosine	2.86	9.10	5.98
N-dimethylglycine	1.94	9.86	5.90
Betaine	1.84	Very large	—

TABLE I—Continued

B. Amino Acids and Peptides with One Amino, One Carboxyl and One or More Phenolic or Sulfhydryl Groups

Substance	pK ₁ ' (COOH)	pK ₂ '	pK ₃ '	pI
Tyrosine	2.20	9.11 (NH ₃ ⁺)	10.07 (OH)	5.66
Diiodotyrosine	2.12	6.48 (OH)	7.82 (NH ₃ ⁺)	4.29
Dibromotyrosine	2.17	6.45 (OH)	7.60 (NH ₃ ⁺)	4.30
Dichlorotyrosine	2.12	6.47 (OH)	7.62 (NH ₃ ⁺)	4.29
3,4-dihydroxyphenylalanine	2.36	8.68 (NH ₃ ⁺)	{ 9.88 (OH)	
			{ 11.68 (OH)	
Glycyltyrosine	2.98	8.40 (NH ₃ ⁺)	10.40 (OH)	5.69
Tyrosyltyrosine	3.52	7.68 (NH ₃ ⁺)	{ 9.80 (OH)	5.60
			{ 10.26 (OH)	
Cysteine	1.96	8.18 (NH ₃ ⁺)	10.28 (SH)	{ 5.07
	1.71*	8.33* (NH ₃ ⁺)	10.78* (SH)	{ 5.02
Cysteinylcysteine	2.65	7.27 (NH ₃ ⁺)	{ 9.35 (SH)	4.96
			{ 10.85 (SH)	

For aspartyltyrosine and tyrosylarginine, see below

C. Substances Containing Two or More Carboxylic Groups and One Basic Group

Substance	pK ₁ ' (COOH)	pK ₂ ' (COOH)	pK ₃ '	pK ₄ '	pI
Aspartic acid	1.88	3.65	9.60 (NH ₃ ⁺)	—	2.77
Glutamic acid	2.19	4.25	9.67 (NH ₃ ⁺)	—	3.22
	2.155*	4.324*	9.960* (NH ₃ ⁺)	—	3.24
β-Hydroxyglutamic acid	2.33	4.24	9.56 (NH ₃ ⁺)	—	3.29
Glycylaspartic acid	2.81	4.45	8.60 (NH ₃ ⁺)	—	3.63
Aspartylglycine	2.10	4.53	9.07 (NH ₃ ⁺)	—	3.31
Aspartylaspartic acid	2.70	3.40	4.70 (COOH)	8.26 (NH ₃ ⁺)	3.04
Glutamylglycine	3.15	—	7.52 (NH ₃ ⁺)	—	—
Glutamylglutamic acid	3.14	4.38	7.62 (NH ₃ ⁺)	—	—
Aspartyltyrosine	2.13	3.57	8.92 (NH ₃ ⁺)	10.23 (OH)	2.85
α-Aminotricarballylic acid	2.10	3.60	4.60 (COOH)	9.82 (NH ₃ ⁺)	2.87
Glycyl-α-amino-tricarbal- lylic acid	2.70	4.10	5.35 (COOH)	8.32 (NH ₃ ⁺)	3.41
Glutathione	2.12	3.53	8.66 (NH ₃ ⁺)	9.12 (SH)	2.83

D. Substances Containing One Carboxyl Group and More than One Basic Group

Substance	pK ₁ ' (COOH)	pK ₂ '	pK ₃ '	pK ₄ '	pI
Histidine	1.82	6.00 (Im)	9.17 (NH ₃ ⁺)	—	7.59
Arginine	2.17	9.04 (NH ₃ ⁺)	12.48 (Guan.)	—	10.76
Ornithine	1.94	8.65 (NH ₃ ⁺)	10.76 (NH ₃ ⁺)	—	9.70
Lysine	2.18	8.95 (αNH ₃ ⁺)	10.53 (εNH ₃ ⁺)	—	9.74
α,β-Diaminopropionic acid	1.33	6.80 (αNH ₃ ⁺)	9.60 (βNH ₃ ⁺)	—	8.20
Histidylhistidine	2.25	5.60 (Im)	6.80 (NH ₃ ⁺)	7.80 (NH ₃ ⁺)	7.30
Histidylglycine	2.40	5.80 (Im)	7.82 (NH ₃ ⁺)	—	6.81
L-Methyl histidine	1.69	6.48 (Im)	8.85 (NH ₃ ⁺)	—	7.67
Carnosine	2.64	6.83 (Im)	9.51 (NH ₃ ⁺)	—	8.17
Anserine	2.64	7.04 (Im)	9.49 (NH ₃ ⁺)	—	8.27
Lysyllsine	1.95	8.17 (NH ₃ ⁺)	9.45 (NH ₃ ⁺)	10.63 (NH ₃ ⁺)	10.04
Phenylalanylarginine	2.66	7.57 (NH ₃ ⁺)	12.10 (Guan.)	—	9.99
Tyrosylarginine	2.65	7.39 (NH ₃ ⁺)	9.36 (OH)	11.62 (Guan.)	8.38
	2.63	7.55 (NH ₃ ⁺)	9.80 (OH)	12.3 (Guan.)	8.68

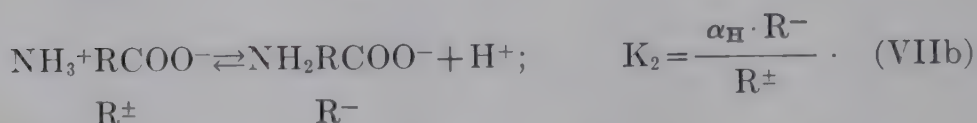
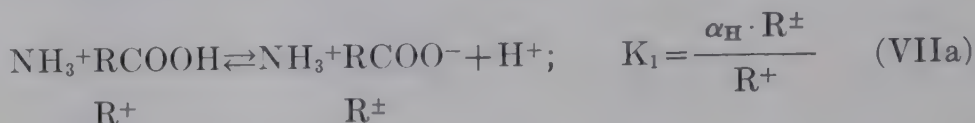
E. Substances Containing Two Carboxylic and Two Amino Groups (Tetrapoles)

Substance	pK ₁ ' (COOH)	pK ₂ ' (COOH)	pK ₃ ' (NH ₃ ⁺)	pK ₄ ' (NH ₃ ⁺)	pI
Cystine	1.65	2.26	7.85	9.85	5.05
	<1.00	1.7	7.48	9.02	4.60
	1.04*	2.05*	8.00*	10.25*	5.03
Lysylglutamic acid	2.93	4.47	7.75	10.50	6.10
Diglycylcysteine	2.71	2.71	7.94	7.94	5.33
Cystinylidiglycine	3.12	3.12	6.36	6.95	4.74
Cystinylididiglycine	3.21	3.21	6.01	6.87	4.61
α-Aspartylhistidine	2.45	3.02	6.82 (Im)	7.98	4.92
β-Aspartylhistidine	1.93	2.95	6.93 (Im)	8.72	4.94

* Denotes pK values calculated by extrapolation of observed measurements to zero ionic strength.

The classical formulation of dissociation is more cumbersome and, indeed, if one works with solutions in which the relation $\alpha_H \cdot \alpha_{OH} = K_w$ is not valid, many problems of acid-base equilibrium become impossible of solution.

The dissociation of amino acids and proteins can be readily represented in terms of the Brönsted concept of acid dissociation. This is illustrated by the equations below



In the above formulation it will be noted that the amino acid cation is considered to be a dibasic acid. Numbering of the acid constants is carried out in sequence starting with the dissociation of the component capable of yielding the greatest number of protons. This, as it turns out, is also the order of progressively decreasing acid strengths of the dissociating groups.

Experimentally, the acid dissociation constants may be evaluated from the titration curve of a substance by considering each separate dissociation step; proceeding in order from the most acid to the most alkaline region. Such a titration curve gives an experimental measure of the number and strengths of the acid groups in a compound. It does not give direct information as to what groups are represented by each of the constants *e.g.*, carboxyl or amino groups. To interpret these dissociation steps in the case of a complex amino acid, *e.g.*, lysine or arginine, requires auxilliary data from structural chemistry.

It is readily deduced that the acid constants are related to the classical and dipolar ion dissociation constants as follows:

$$K_1 = K_A = \frac{K_w}{K_b} \quad (VIIIa)$$

$$K_2 = \frac{K_w}{K_B} = K_a \quad (VIIIb)$$

The apparent dissociation constants of amino acids, peptides, and related substances are given in Table I in terms of the Brönsted acid constants. Representative titration curves of amino acids are given in Figs. 2 to 4.

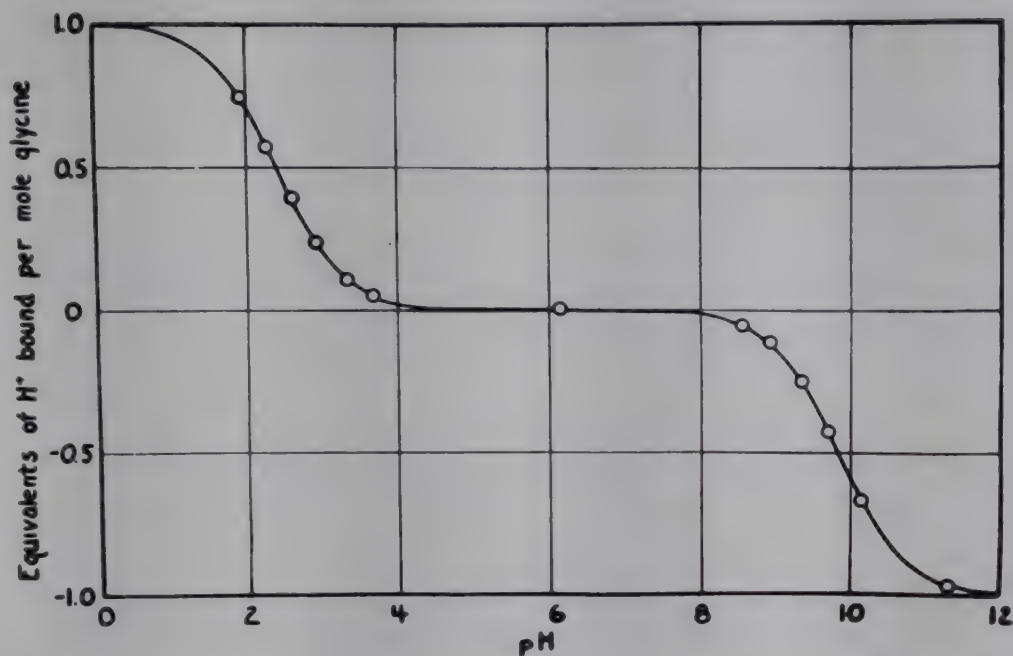


FIG. 2. Dissociation curve of glycine at 18° and ionic strength 0.1 *M*. recalculated from Sørensen's pH measurements by Hitchcock. Hitchcock, D. I. in Schmidt, C. L. A.: *Chemistry of the Amino Acids and Proteins*. p. 608.

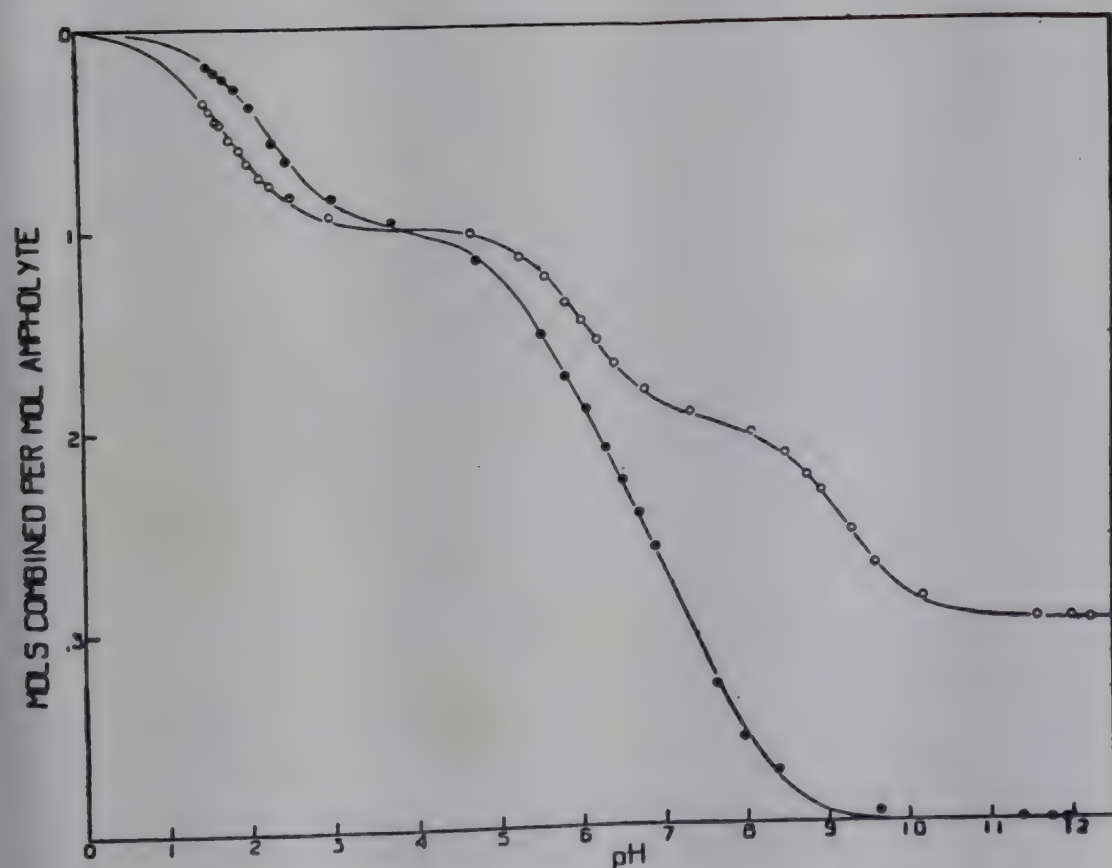


FIG. 3. Dissociation curves of histidine (○) and histidyl-histidine (●). The curves are calculated from the titration constants given in Table I, the points are experimental. Greenstein, J. P.: *J. Biol. Chem.*, 93:479 (1931).

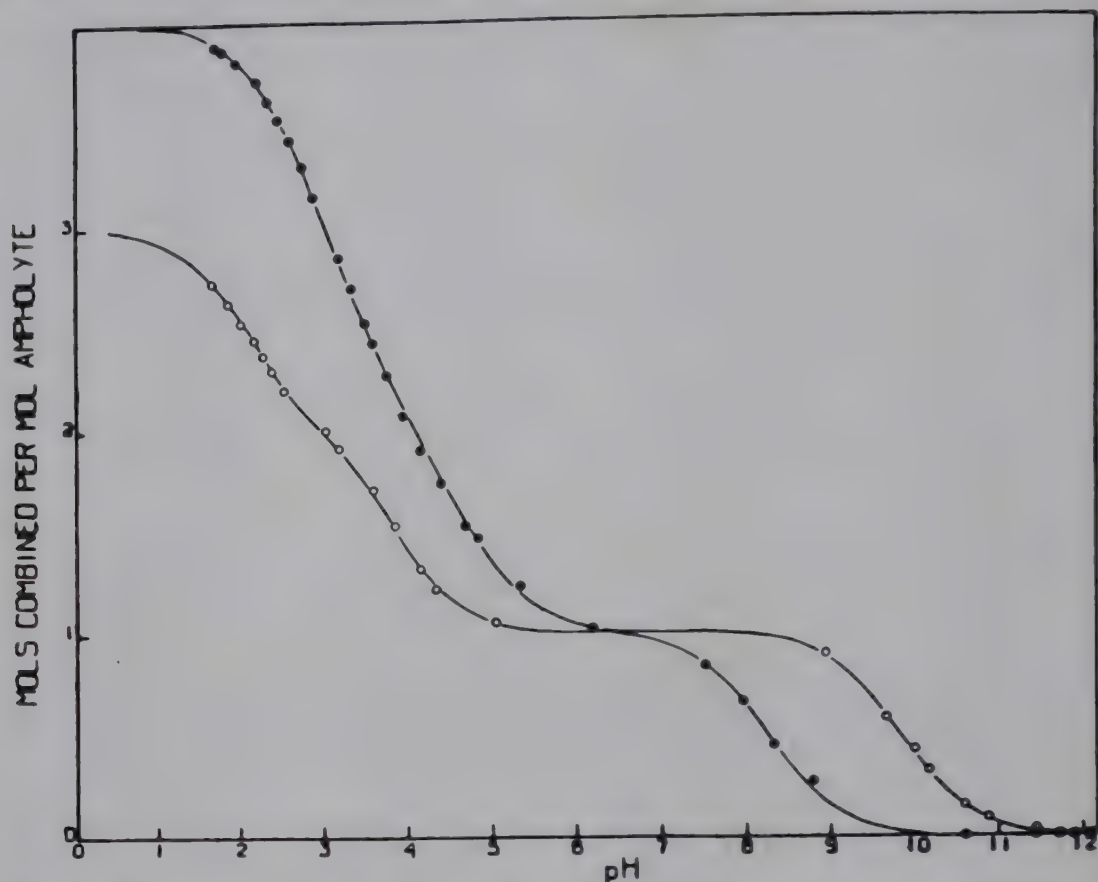


FIG. 4. Dissociation curves of aspartic acid (○) and aspartyl aspartic acid (●). The curves are calculated from the titration constants given in Table I, the points are experimental. Greenstein, J. P.: *J. Biol. Chem.*, 93:479 (1931).

IV. THE ISOELECTRIC STATE

1. Equation of the Isoelectric Point

The isoelectric state is important because many properties of aqueous solutions of ampholytes exhibit minima or maxima in this region. Among the properties that exhibit minima are: (a) solubility; (b) membrane potential; (c) osmotic pressure; (d) swelling of protein gels; (e) optical rotation; and, (f) diffusion. Properties that show maxima are; (a) degree of dissociation; (b) electrostriction; (c) light scattering; and, (d) stability, *i.e.*, resistance to denaturation.

The isoelectric point may be defined as the pH at which the net electrical charge of the ampholyte is zero. An important consequence of this state is that there will be no migration of the ampholyte in an electric field. A fundamental method of determining the isoelectric points of proteins is to find the pH region of zero electrophoresis.

The equation for the isoelectric pH of a simple amino acid may

be derived by multiplying together equations VIIa and VIIb. This yields

$$K_1 K_2 = (\alpha_H)^2 \frac{R^-}{R^+}.$$

At the isoelectric point $R^- = R^+$. Now designating the value of α_H at the isoelectric point by I , there is obtained

$$I = \sqrt{K_1 K_2}. \quad (\text{IXa})$$

In terms of pH this yields the equation

$$pI = 1/2(pK_1 + pK_2). \quad (\text{IXb})$$

To extend the same form of treatment to more complex ampholytes let us take the case of one that is dibasic and diacid and which yields the ionic and molecular species, R^{++} , R^+ , R , R^- and R^{--} .

The dissociation equations of such an ampholyte are

$$\begin{aligned} K_1 &= \frac{\alpha_H \cdot R^+}{R^{++}}, & K_2 &= \frac{\alpha_H \cdot R}{R^+}, \\ K_3 &= \frac{\alpha_H \cdot R^-}{R}, & K_4 &= \frac{\alpha_H \cdot R^{--}}{R^-}. \end{aligned} \quad (\text{X})$$

In the isoelectric state the average net charge per ampholyte molecule will be zero, so that in terms of equivalent concentrations,

$$2R^{++} + R^+ = R^- + 2R^{--}.$$

Inserting the appropriate terms from equation X and designating the isoelectric value of α_H by I there is obtained

$$\frac{2I^2}{K_1 K_2} + \frac{I}{K_2} = \frac{K_3}{I} + \frac{2K_3 K_4}{I^2} \quad (\text{XI})$$

or

$$\frac{I}{K_2} \left(1 + \frac{2I}{K_1} \right) = \frac{K_3}{I} \left(1 + \frac{2K_4}{I} \right) \quad (\text{XIa})$$

or

$$I^2 = K_2 K_3 \frac{1 + (2K_4/I)}{1 + (2I/K_1)}. \quad (\text{XIb})$$

For any number of ionizing groups, equation XI takes the general form

$$\begin{aligned} \frac{I}{K_m} \left(1 + \frac{2I}{K_{m-1}} + \frac{3I^2}{K_{m-2} K_{m-1}} + \dots \right) \\ = \frac{K_{m+1}}{I} \left(1 + \frac{2K_{m+2}}{I} + \frac{3K_{m+2} K_{m+3}}{I^2} + \dots \right). \end{aligned} \quad (\text{XII})$$

In the above equation, K_m is the constant representing the dissociation step between the univalent cation and uncharged ampholyte, the constants of lower value than m represent the dissociation steps of the cationic groups and the constants greater than m the dissociation steps of the anionic groups.

If K_{m-1} is much greater than $2I$ and $2K_{m+2}$ is much less than I , equation XII reduces to IXa, the equation for a simple ampholyte with one acid and one basic group. In other words, the isoelectric point can be calculated, to a reasonable approximation, from the values of the dissociation constants representing the acid dissociation steps of the univalent cation and of the undissociated ampholyte.⁵

As a numerical illustration of the application of the above equations we may take the example of the amino acid cystine cited by Edsall (12). The dissociation constants for this amino acid are: $pK_1 = 1.04$, $pK_2 = 2.05$, $pK_3 = 8.00$, and $pK_4 = 10.25$.

Making use of equation IXb as an approximation yields:

$$pI = 1/2(2.05 + 8.00) = 5.03$$

or

$$\alpha_H = 10^{-5.03}.$$

Substituting this approximate value and the numerical values of the constants in equation XIb there is obtained

$$I^2 = K_2 K_3 \frac{(1.000012)}{(1.0002)}.$$

It is obvious that the solution will yield a result indistinguishable from the result obtained with equation IXb.

2. Span of Isoelectric Zones

The values of pK_1 and pK_2 are so widely separated in most amino acids that the isoelectric region is a broad zone. In solutions of

⁵ A more general mathematical analysis of the theory of the isoelectric point has been published by Hill (28). A useful approximate equation for the isoelectric point of a polyvalent ampholyte in terms of the classical dissociation constants was derived by Levene and Simms (38). It is

$$I = \sqrt{\frac{\sum K_a}{\sum K_b} K_w}. \quad (\text{XIII})$$

glycine the concentration of R^+ and R^- is less than 1% of R^\pm over the pH range between 4.3 and 7.7.

Hitchcock (27) has shown that the breadth of the isoelectric zone may be determined by defining it as the difference in pH between two points on the opposite branches of the ρ -pH curve where ρ , the dissociation residue, has the same value, which is specified as a definite large fraction, f , of its maximal value. The dissociation residue is defined by the equation.

$$\frac{1}{\rho} = 1 + \frac{\alpha_H}{K_1} + \frac{K_2}{\alpha_H}$$

and its maximal value, at the isoelectric point, is given by

$$\frac{1}{\rho_{\max}} = 1 + 2\sqrt{K_2/K_1}.$$

This leads to the relation defining f given below

$$f = \frac{1 + 2\sqrt{K_2/K_1}}{1 + \frac{\alpha_H}{K_1} + \frac{K_2}{\alpha_H}}. \quad (\text{XIVa})$$

To simplify equation XIV there is introduced a new variable, q , defined as

$$q = \frac{1-f}{f} \sqrt{\frac{K_1}{K_2} + \frac{2}{f}}. \quad (\text{XIVb})$$

This yields the relation

$$q = \frac{\alpha_H}{I} + \frac{I}{\alpha_H}.$$

Upon solving for α_H there is obtained

$$\alpha_H = \frac{qI}{2} (1 \pm \sqrt{1 - 4/q^2}). \quad (\text{XV})$$

The breadth of the isoelectric zone in pH units is given by the difference between the logarithms of the two values of α_H obtained from equation XV. This leads to

$$\Delta\text{pH} = \log \frac{1 + \sqrt{1 - 4/q^2}}{1 - \sqrt{1 - 4/q^2}}. \quad (\text{XVI})$$

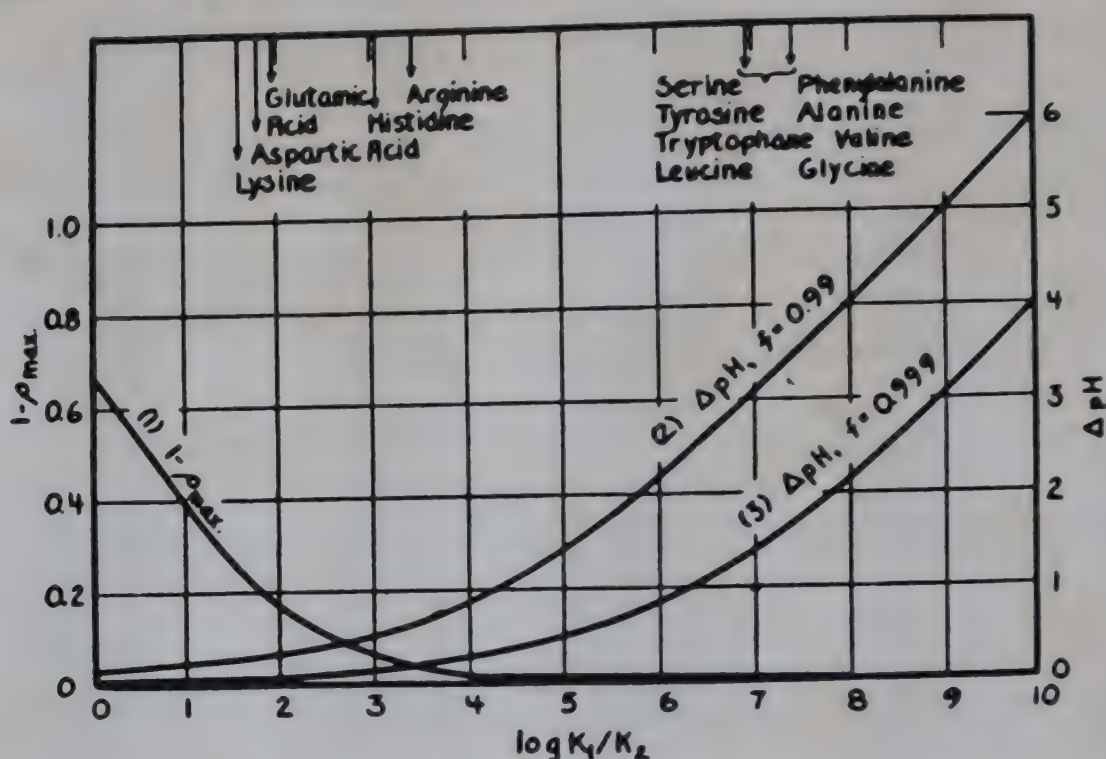


FIG. 5. Curve 1 shows values of $1 - \rho_{\max}$, the fraction of a simple ampholyte ionized as cations and anions at the isoelectric point, as a function of the logarithm of the ratio of its dissociation constants. Curves 2 and 3 show ΔpH , the breadth of the isoelectric zone of a simple ampholyte, so defined that the electrically neutral fraction shall have 99.0 and 99.9% of its maximum value. Hitchcock, D. I.: *J. Biol. Chem.*, 114:373 (1936).

Curves showing the breadth of the isoelectric zones of amino acids at several values of f are plotted in Fig. 5.

The isoelectric point depends on the values of the dissociation constants of the acid and basic groups of an ampholyte and these will vary with the nature of the solution, particularly the ionic strength.⁶ Consequently, the isoelectric point will also vary with the ionic strength of the solution. Smith (64) found that the isoelectric point of egg albumin varied inversely as the ionic strength of the buffer used and directly with concentration of the protein.

V. THE EQUILIBRIUM BETWEEN DIPOLAR IONS AND UNCHARGED AMPHOLYTES

Evidence for the dipolar ion structure of the amino acids has already been presented. This does not preclude the possibility that

⁶ The ionic strength is defined by the equation

$$\mu = \frac{m_+ v_+^2 + m_- v_-^2}{2}$$

where m_+ and m_- represent the molal concentration of cation and anion respectively of an electrolyte and v_+ and v_- the valency, respectively (39).

some fraction of the amino acid may exist as the uncharged ampholyte, since the two forms are in mobile equilibrium with each other. This equilibrium can be represented by the equation

$$K_z = \frac{R^\pm}{R} \quad (\text{XVII})$$

By a round about method the value of K_z can be derived approximately from the relative dissociation constants of the amino acids and of their esters (see references 12, 62).

The relation obtained is

$$K_z = \frac{K_1}{K_E} - 1.$$

where K_E represents the dissociation constant of the amino acid ester. However, if K_1 is very much larger than K_E , the above equation reduces to

$$K_z = \frac{K_1}{K_E} \quad \text{or} \quad -\log K_z = pK_E - pK_1.$$

K_z is of the order of magnitude of 10^5 for most amino acids and the quantity of uncharged ampholyte normally present in their solutions is vanishingly small. Only in the case of the aminobenzoic acids, where K_z is -0.70 , $+0.36$, and -0.87 respectively for the *o*, *m*, and *p* compounds, does the uncharged ampholyte become of comparable concentration with the dipolar ion.

VI. EFFECT OF pH ON THE SOLUBILITY OF AMPHOLYTES

The pH is the most important variable affecting the solubility of aqueous solutions of amino acids and proteins that are slightly soluble at the isoelectric point. The problem becomes one of calculating the solubility of an ampholyte of low solubility, which forms soluble salts with acid or alkali, from the dissociation constants and the pH of the solution. The simplest case is derived as follows:

The total solubility will be represented by

$$S = S_0 + S^+ + S^-$$

where S represents the total concentration of ampholyte in solution, S_0 the isoelectric molecules, S^+ the cations, and S^- the anions. From the dissociation equations VIIa and VIIb we may write

$$S = S_0 + \frac{\alpha_H S_0}{K_1} + \frac{K_2 \cdot S_0}{\alpha_H}$$

or

$$S = S_0 \left[1 + \frac{\alpha_H}{K_1} + \frac{K_2}{\alpha_H} \right]. \quad (\text{XVIII})$$

This equation gives the solubility of the ampholyte in terms of the solubility of the uncharged molecule and the hydrogen ion activity of the solution.

For more complex situations the solubility may be represented by

$$S = S_0 + (p^+ + p^-) + (p^{++} + p^{--}) + \cdots + (p^{n+} + p^{m-})$$

where the maximum valency of the cations is represented by n^+ and the anions by m^- . Upon substituting for the concentrations of each of the ions the mass law expressions in terms of α_H and S_0 , in the manner indicated by equation XII there is obtained the solubility equation.

$$S = S_0 \left[1 + \left(\frac{\alpha_H}{K_n} + \frac{K_{n+1}}{\alpha_H} \right) + \left(\frac{\alpha_H^2}{K_n K_{n-1}} + \frac{K_{n+1} K_{n+2}}{\alpha_H^2} \right) + \cdots + \left(\frac{\alpha_H^n}{K_n K_{n-1} K_{n-2} \cdots K_1} + \frac{K_{n+1} K_{n+2} K_{n+3} \cdots K_{n+m}}{\alpha_H^m} \right) \right]. \quad (\text{XIX})$$

The effect of pH on the solubility of amino acids has been determined by Hitchcock (26) for tyrosine and by Dalton, Kirk and Schmidt (13) for diiodotyrosine. Green (19) has studied the influence of the pH on the solubility of hemoglobin and egg albumin. The pH-solubility curves of the amino acids, tyrosine and diiodotyrosine, are reproduced in Fig. 6 and those of the proteins, hemoglobin, and egg albumin, in Fig. 7.

VII. TITRATION IN MIXED SOLVENTS

The titration of amino acids in solutions containing formaldehyde, ethanol, acetone, and acetic acid has considerable utility

1. Formaldehyde Titration

The oldest and most important of these reactions in mixed solvents is the formaldehyde titration. The value of this is that the formaldehyde greatly decreases the apparent basicity of the amino

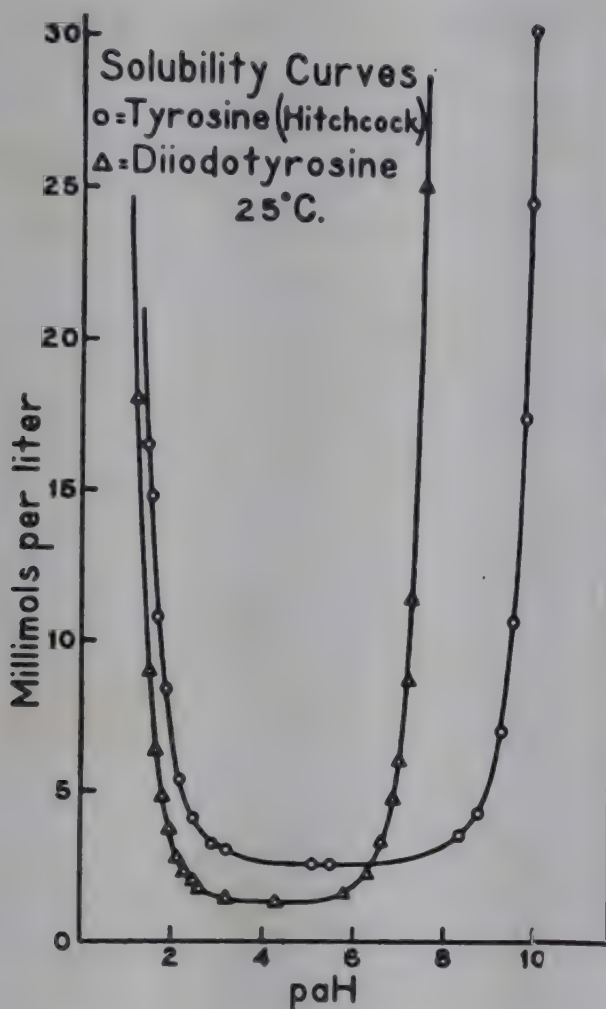


FIG. 6. Dalton, J. B., Kirk, P. L., and Schmidt, C. L. A.:
J. Biol. Chem., 88:589 (1930).

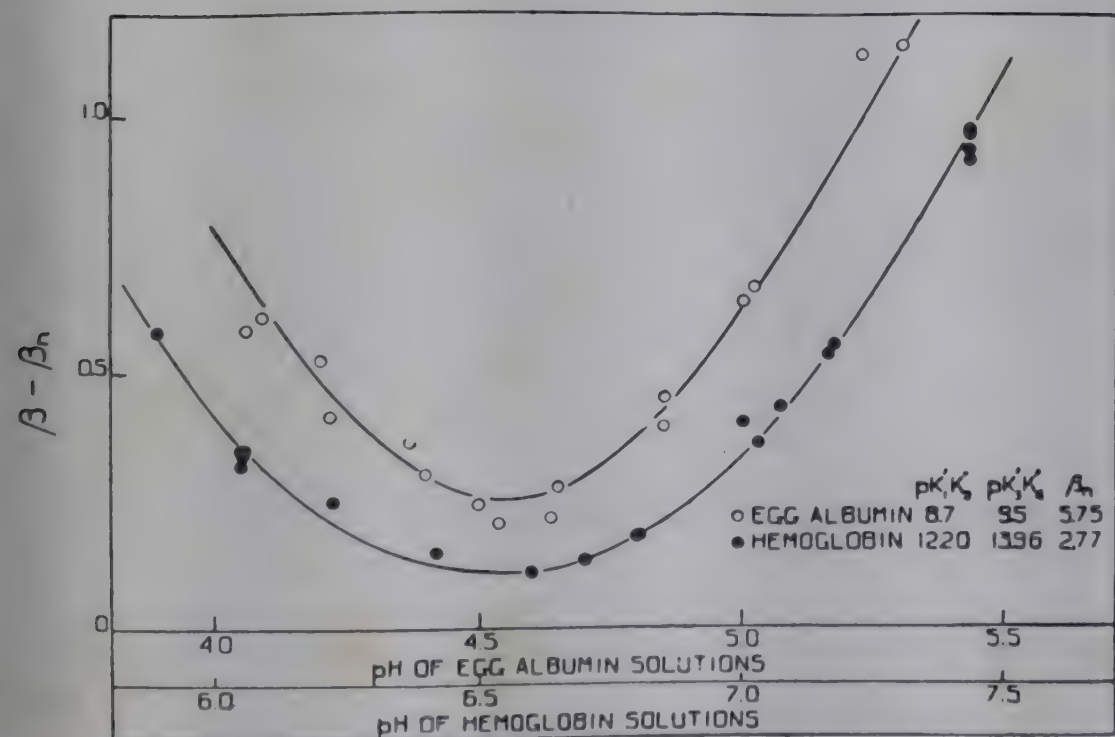


FIG. 7. The solubility of hemoglobin and of egg albumin in concentrated salt solutions of varying pH. Green, A. A.: *J. Biol. Chem.*, 93:517 (1931).

group without appreciably altering the degree of alkalinity required to cause an indicator such as phenolphthalein to change color.

Birch and Harris (3) and Harris (24, 25) showed that in the titration curve of an amino acid, the portion representing the carboxyl dissociation was virtually unchanged while the dissociation of the amino group was markedly shifted to the acid side. In general, the pK_2 values of the monoamino monocarboxylic acids progressively decrease with increasing formaldehyde concentration (69).

The interpretation of the reaction as proposed by Levy (36) is that (a) only the uncharged amino group reacts with formaldehyde, (b) amino groups can combine rapidly and reversibly with either one or two molecules of formaldehyde, and (c) the formaldehyde addition products are such weak bases that their basicity may be neglected in considering the dissociation equilibria.

2. Alcohol and Acetone Titrations

The effect of alcohol and acetone have a somewhat similar explanation. Due to the decrease in the dielectric constants of these solutions, the dissociation of the ionizable groups in the amino acids are markedly altered. In the case of the monoamino monocarboxylic acids, the pK_1 is markedly increased while the pK_2 value is changed but little⁷ (30).

There are, in addition, shifts in the dissociation of the indicators employed in these titrations.

3. Glacial Acetic Acid

In glacial acetic acid, certain acids (sulfuric, hydrobromic, perchloric), become super acids. Amino acids dissolved in glacial acetic acid exhibit a large increase in their apparent basicity and can be readily titrated with the above acids using crystal-violet, α -naphthol-benzene, or benzoylauramine as indicators (53).

Typical titration curves demonstrating these properties are given in Figs. 8–12. Dissociation constants of amino acids in the presence of formaldehyde and of ethanol are given in Tables II and III.

VIII. AMPHOTERIC PROPERTIES OF PROTEINS

The amphoteric properties of proteins may be expected to be a composite of the effects of the amino acid residues they contain. Over the pH range suitable for protein titration (pH 1–13), certain

⁷ The dissociation constants of a number of amino acids in 20 percent dioxane-water mixtures has been determined by Duggan and Schmidt (15).

groups are known not to function as either acids or bases.⁸ These groups are the peptide linkage, the amide groups of asparagine and glutamine, and the aliphatic hydroxyl groups of serine, threonine and hydroxyproline. The peptide linkage, in general, eliminates the α -amino and α -carboxyl groups of the amino acids from playing

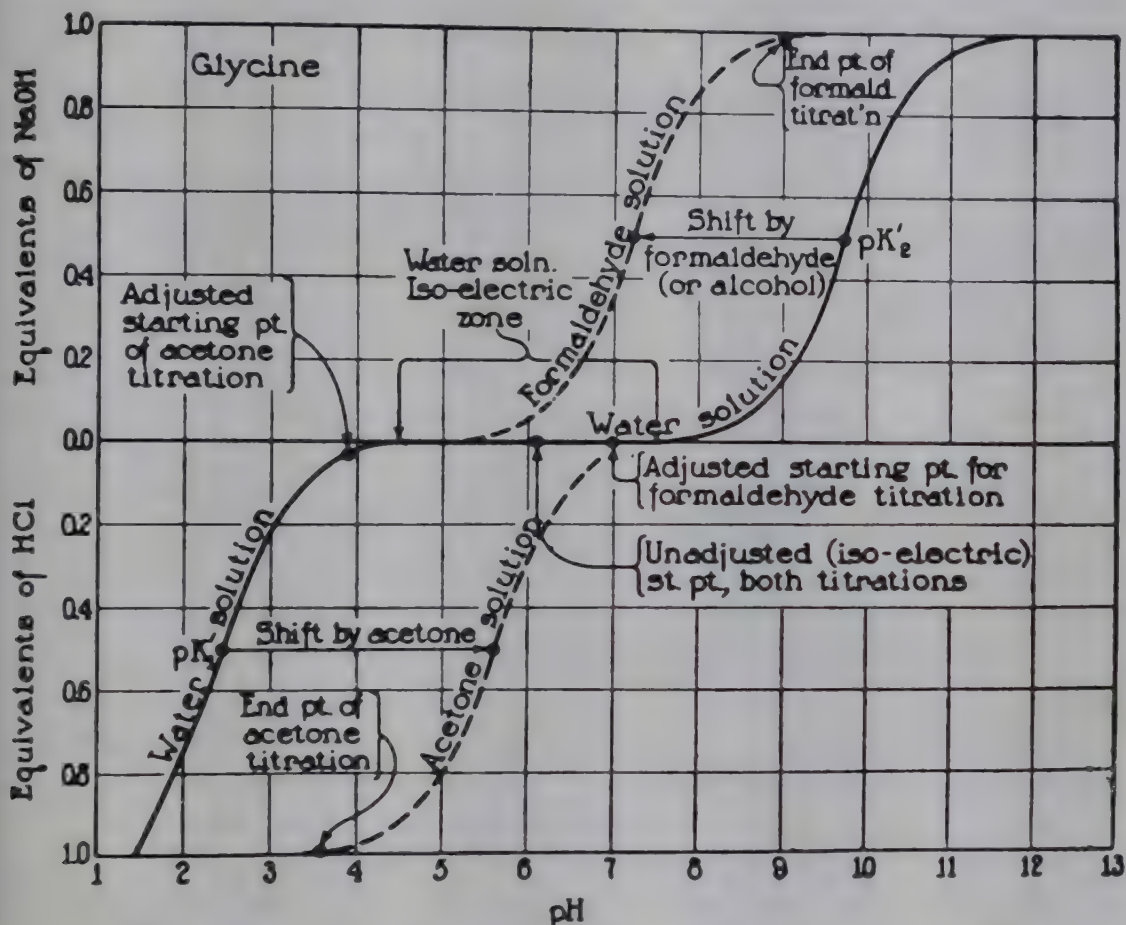


FIG. 8. Titration curve of glycine. From Van Slyke, D. D., and Kirk, E.: *J. Biol. Chem.*, 102:651 (1933).

a role in the amphoteric dissociation of proteins. Occasionally, one of the above groups may occur as the terminus of a peptide chain, and thus contribute to a slight degree to the total acid or base binding.

From their known composition, it appears plausible that aspartic and glutamic acids furnish the titratable carboxyl groups in proteins, tyrosine supplies an acid phenolic, and cysteine a thiol group; the basic groups of the protein are derived from the guanidino group of arginine, the imidazole group of histidine, and the ϵ -amino group of lysine. Ornithine, if it is present in proteins, which has

⁸ Cystine may play a special role in the acid-base binding of proteins since only two of its four ionizable groups necessarily occur in peptide linkage.

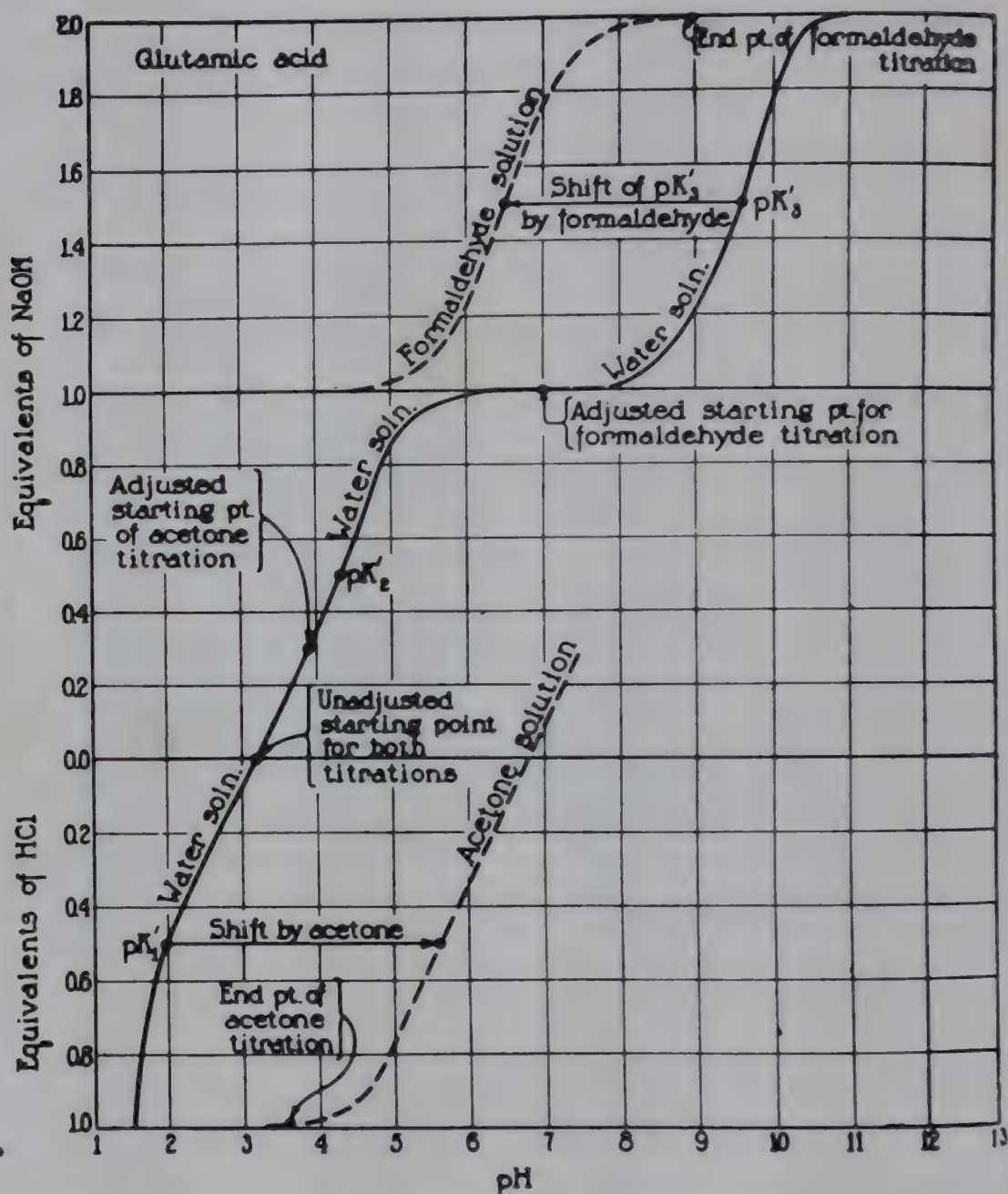


FIG. 9. Titration curve of glutamic acid. From Van Slyke, D. D., and Kirk, E.: *J. Biol. Chem.*, 102:651 (1933).

never been demonstrated, would also contribute an amino group. In addition, in conjugated proteins the prosthetic group may be either acidic or basic and contribute to the acid or base binding. This is exemplified by the phosphate radical of casein and the heme of hemoglobin.

1. Characterization of the Ionizing Groups in a Protein

The dissociation constants and heats of ionization of the dissociable groups that may be present in proteins are given in Table IV.

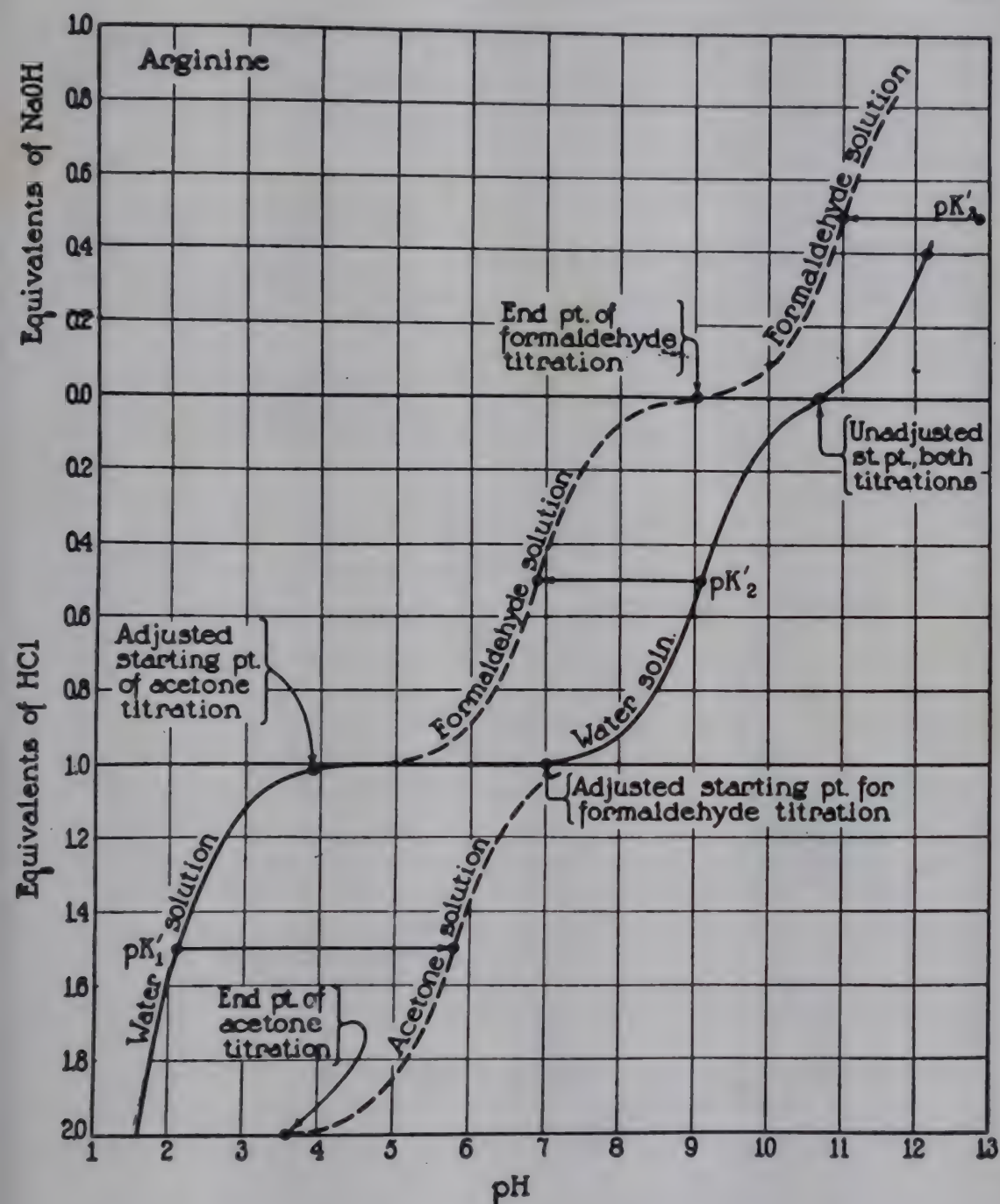


FIG. 10. Titration curve of arginine. From Van Slyke, D. D., and Kirk, E.: *J. Biol. Chem.*, 102:651 (1933).

A number of methods are available for characterizing the nature of the ionizing groups in the various pH zones of the titration curve of a protein. The gist of these methods are presented below. A more complete discussion is given by Edsall (12).

The outstanding method is a comparison of the titration regions of a protein with that expected from the pK values of the ionizable groups of the amino acid residues of the protein. The known possibilities are listed in Table IV. This procedure may be falsified by

shifts in the pK values owing to the effects of neighboring groups on the dissociation. Ordinarily these are small, but in several instances shifts in the dissociation region of as much as 2 pH units have been reported.

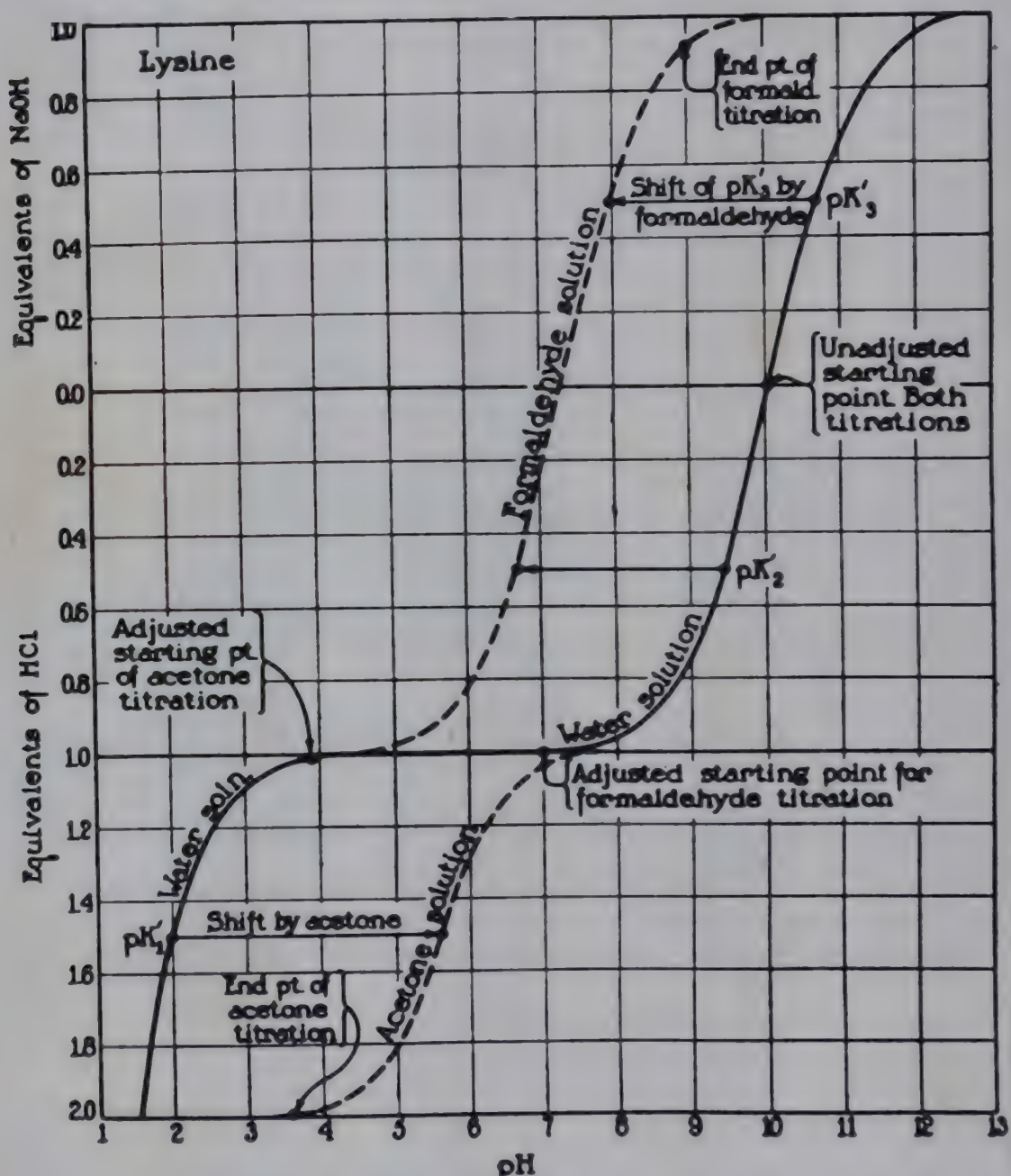


FIG. 11. Titration curve of lysine. From Van Slyke, D. D., and Kirk, E.: *J. Biol. Chem.*, 102:651 (1933).

Determination of the heats of ionization is a valuable supplement to the dissociation constant method in affording a clue to the nature of the ionizable groups. These may be evaluated by carrying out the titration curve of a protein at two or more different temper-

atures and calculating the heats of ionization by means of the van't Hoff equation.

Changes in the titration curve produced by formaldehyde and, if interpreted with caution, by alcohol afford evidence of the nature

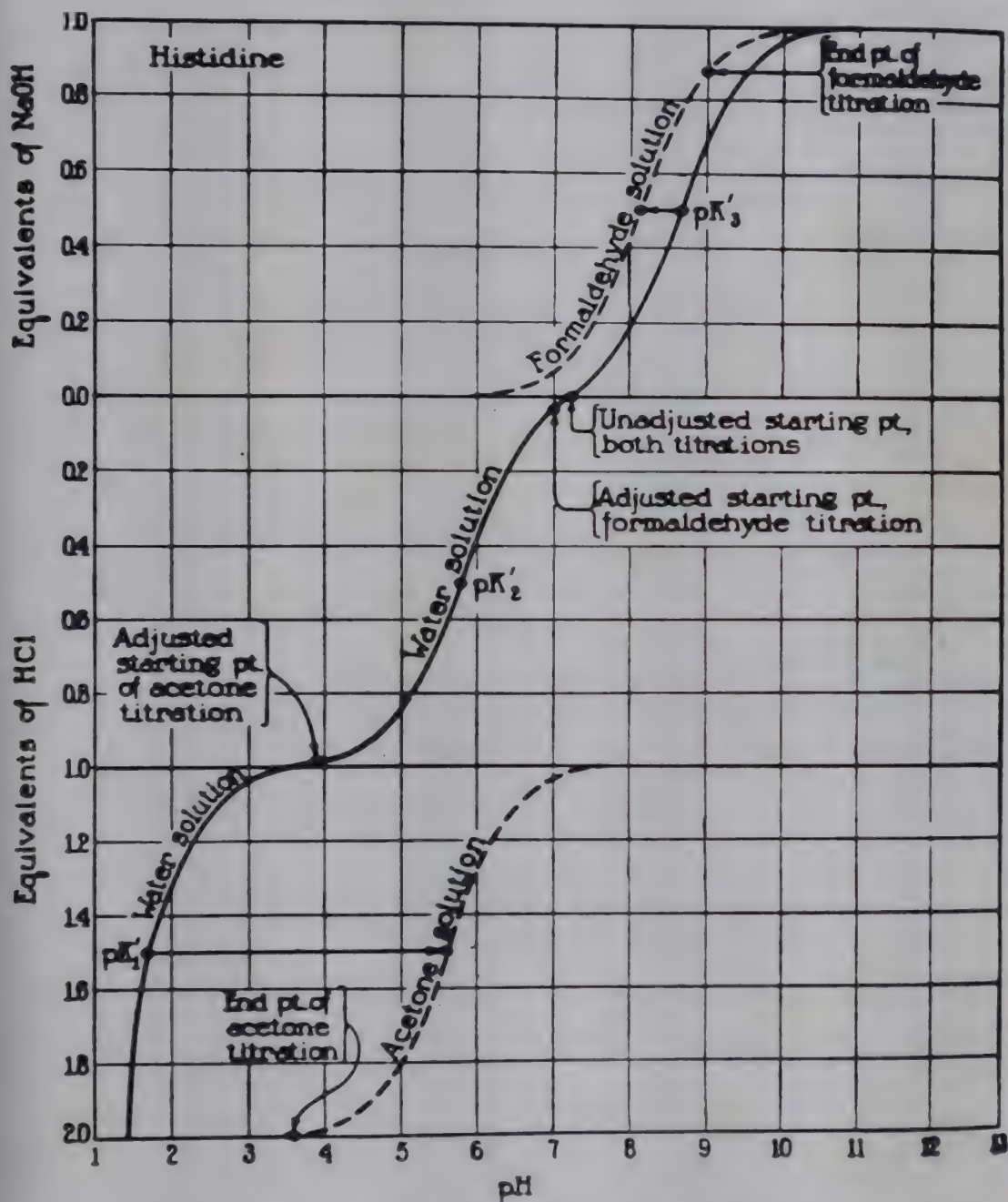


FIG. 12. Titration curve of histidine. From Van Slyke, D. D., and Kirk, E.: *J. Biol. Chem.*, 102:651 (1933).

of the titratable groups. Formaldehyde markedly shifts the titration of the cations of the amino and imidazole groups to lower pH regions while the titration of carboxylic and phenolic hydroxyl groups remain virtually unchanged. Alcohol commonly produces

TABLE II
IONIZATION CONSTANTS OF THE AMINO ACIDS IN AQUEOUS
FORMALDEHYDE SOLUTION^a.

Amino Acid	Mole Per Cent Formaldehyde				
	0.99	3.95	5.60	10.0	17.9
DL-Alanine	8.36	7.42	6.96 ^b	6.56	6.10
L-Arginine		3.45 ^c	3.40 ^d		
L-Aspartic acid			7.21 ^d	≍ 3.8 ^e 6.85 ^f	
L-Glutamic acid			6.91 ^d	≍ 4.2 ^e 6.8 ^f	
Glycine	7.16	6.08	5.92 ^b	5.34	5.04
L-Histidine		7.90 ^e	7.90 ^d		
L-Hydroxyproline			7.19 ^d		
L-Leucine	8.44	7.50	6.92 ^d	6.92	6.20
DL-Leucine	8.44	7.48		6.60	6.20
L-Lysine		7.35 ^e	7.15 ^d		
L-Norleucine	8.42	7.48		6.61	
DL-Norleucine	8.42	7.48	7.10 ^b	6.61	6.21
L-Phenylalanine			6.62 ^d	5.9 ^e	
DL-Phenylalanine	8.09	7.16	6.80 ^b	6.35	6.13
L-Proline			7.78 ^d		
DL-Serine	6.66	5.74	5.63 ^b		4.94
L-Tryptophan			6.88 ^d		
L-Tyrosine			7.50 ^d	6.2 ^e > 9 ^f	
DL-Valine	8.52	7.65	7.47 ^b		6.52

^a Dunn and Weiner (17), pK₂ at 22°.

^b Dunn and Loshakoff (16), pK₂ at 22°.

^c Levy (36) pK₂ at 30° for arginine and pK₃ at 30° for histidine and lysine.

^d Levy and Silberman (37), pK₂ at 30°, pK₃ at 30° for histidine and lysine.

^e Harris (24), pK₂ at 25° for aspartic acid, glutamic acid, phenylalanine and tyrosine.

^f Harris (24), pK₃ at 30° for aspartic acid, glutamic acid, and tyrosine.

(Dunn, M. S., *Handbook of Chemistry and Physics*, Cleveland, Ohio, 26th Ed., 1942.)

TABLE III
IONIZATION CONSTANTS OF THE AMINO ACID IN AQUEOUS
ETHANOL SOLUTIONS (30)

Amino Acid	pK ₁	pK ₂	pK ₃	Volume Per Cent Ethanol	Tempera- ture°
Alanine	3.55	10.02		72	25
Arginine	3.34	9.40	14.1	72	25
Aspartic acid	2.85	5.20	10.51	72	25
Glutamic acid	3.16	5.63	10.75	72	25
Glycine	2.66	9.82		10	19.5 (51)
	2.96	9.76		40	19.5 (51)
	3.46	9.82		72	25
	3.79	9.99		90	19.5 (51)
Histidine	3.00	5.85	9.45	72	25
Isoleucine	3.69	9.81		72	25
Lysine	2.75	8.95	10.53	48	25
	3.56	8.95	10.49	84	25
Proline	3.04	10.55		72	25
Valine	3.60	9.73		72	25

(Compiled by Dunn, M. S., *Handbook of Chemistry and Physics*, Cleveland, Ohio, 26th Ed., 1942.)

an increase in the pK value of the carboxyl group and decreases the acid pK values of the amino, imidazole and guanidine groups. The denaturing effect of alcohol and irregularities in the ionization shifts, due to the total electrical state of the protein, may invalidate the deductions to be drawn. Alcohol is particularly useful in characterizing the prolamines.

Elimination or masking of ionizable groups by specific chemical reactions may also be useful in isolating segments of the titration curve of a protein. Thus the ϵ -amino group of lysine may be eliminated by treatment with nitrous acid and amino and phenol

TABLE IV
IONIZING GROUPS OF PROTEINS (8)

Group	Amino Acid	pK ₀ '	Q'	$\Delta pK'$ ($\Delta t = 10^\circ$)
α -Carboxyl		3.5	calories 0	0
Carboxyl	{ Aspartic acid	4.0	0	0
	{ Glutamic acid			
Imidazole	Histidine	7.0	7,000	-0.17
α -Amino		8.0	10,500	-0.26
Amino	Lysine	10.0	10,500	-0.26
Phenolic	Tyrosine	10.0	6,000	-0.15
Sulfhydryl	Cysteine	10.0	?	?
Guanidine	Arginine	12.5	12,500	-0.31

(Cannan, R. K., Chem. Revs., 30: 395 (1942).)

groups may be made non-reactive ionically by acetylation with ketene. Phenolic groups may also be characterized by iodination.

2. Isoelectric and Isoionic Points

The most important amphoteric constant of a protein is the isoelectric point. This is quite sharply defined in most proteins, in contrast to that of many of the amino acids. The isoelectric point is the pH region at which the net electrical charge of a protein is zero. This point may be determined with precision by the Tiselius electrophoresis method (68). A list of the isoelectric points of certain important proteins is contained in Table II, Chapter VIII.

The net charge on a protein may be affected by interaction with other ions than hydrogen or hydroxyl. This leads to the concept of the *isoionic point* (66), which may be defined as the pH at which the number of protons combined on the basic groups (NH_2 , etc.) is equal to the number dissociated from the acidic groups ($-COOH$ etc.). The total charge is zero only if the protein combines with no other ions than hydrogen ion, in which case the isoionic and iso-

electric points are identical. If other ions do react the protein will carry an electric charge at the isoionic point.

This constant may be determined in a number of ways (8). If the protein can be isolated essentially salt-free, in the case of the globulins by spontaneous precipitation at low electrolyte concentration and with more soluble proteins (albumins) by electrodialysis, the isoionic point practically coincides with the pH of solution of the electrolyte-free protein, provided the concentration of protein is not less than one per cent and the pH falls between 4.5 and 9.5.

The isoionic point may also be located from the titration curves of a protein at different protein concentrations. At low electrolyte concentration, the slopes of the titration curves will vary with the concentration of protein but will all intersect at the isoionic point.

IX. PROTEIN DISSOCIATION CURVES

1. Acid-Base Combining Capacity

The reaction of a protein with hydrogen and hydroxyl ions may be determined from its titration curve with a strong acid or a base.

This is carried out by determining the pH, with a hydrogen or glass electrode, of solutions containing a fixed quantity of protein and varying amounts of acid or base. The hydrogen or hydroxyl neutralized by the protein, designated by the letter h , is computed by subtracting the amount of free hydrogen or hydroxide ion in each solution from the total acid or base added. The quantity h is taken to be positive when acid is bound and negative when base is bound.

Cannan (8) points out that the logical origin of the h -scale is the isoionic point rather than the isoelectric point. When h is measured from the isoionic point, it represents the difference between the total acid or base added to the protein solution and the free hydrogen or hydroxide ion left in bringing the solution from the isoionic point to the desired pH value. The free hydrogen or hydroxide ion is usually determined by measuring the amount of acid or base which must be added to the same quantity of protein-free solution to attain the same change in pH. It is desirable that the protein and protein-free solution have, as closely as possible, the same electrolyte composition. Except at the extremes of pH, the quantities of free hydrogen or hydroxide ion are relatively small, and the values of h can be determined with considerable accuracy. At either extreme of the pH range, h becomes a small difference between the

total and free acid or base and becomes difficult to estimate accurately. This makes the evaluation of the maximum acid- and base-binding capacity of proteins difficult. Much of the difficulty is due to the unknown alterations in the activity coefficient of the hydrogen ion produced by the protein and electrolytes of the solution. The uncertainties of calculation may be minimized by conducting the titrations on solutions containing a relatively high concentration of protein and a large excess, at a constant ionic strength, of a neutral salt like sodium or potassium chloride. The most reproducible values are obtained in this manner.

Examination of the strengths of the probable ionizable groups of proteins (Table IV) indicates that all the carboxyls in the protein molecule remain undissociated and all the cationic groups carry positive charges between pH 1 and 2. Thus the acid bound by proteins may be expected to attain a maximum value, independent of the pH, between pH 1 and 2. This coincides with experimental observation. In an alkaline solution, however, an appreciable number of the guanidino groups of the arginine residues still retain their positive charge even at a pH of 13. For this reason no sharply defined maximum value of the base bound by a protein is to be expected.

2. Effect of Denaturation

The number of acid- and base-combining groups derived from the titration curve of a protein may be falsified by the irreversible denaturation reactions which are prone to occur at the extremes of pH at which the maximum combining capacities are measured. While denaturation does not necessarily alter the ionizable groups, it often does. Thus, the acid-binding of egg albumin is not altered by denaturation in dilute acid but the base-binding capacity changes at a pH of around 12 (8). The irreversible reaction produced by hydrogen or hydroxyl ions may often be detected by drifts in the measured potentials with time. This is not an invariable test, particularly in well buffered regions.

3. Interpretation of Protein Dissociation Curves

The titration curves of three well characterized, crystalline proteins, carboxyhemoglobin, egg albumin and β -lactoglobulin are shown in Figs. 13-15.

The titration curves of individual proteins, as is seen from the above figures, have much in common. The slopes of the curves are markedly affected by electrolytes in a manner which can be ap-

proximately represented as a function of the ionic strength. The dissociation curve of a protein is usually divisible into three distinct regions; one from the most acid region to about pH 5, the second from pH 5 to 9 and the third from pH 9 on.

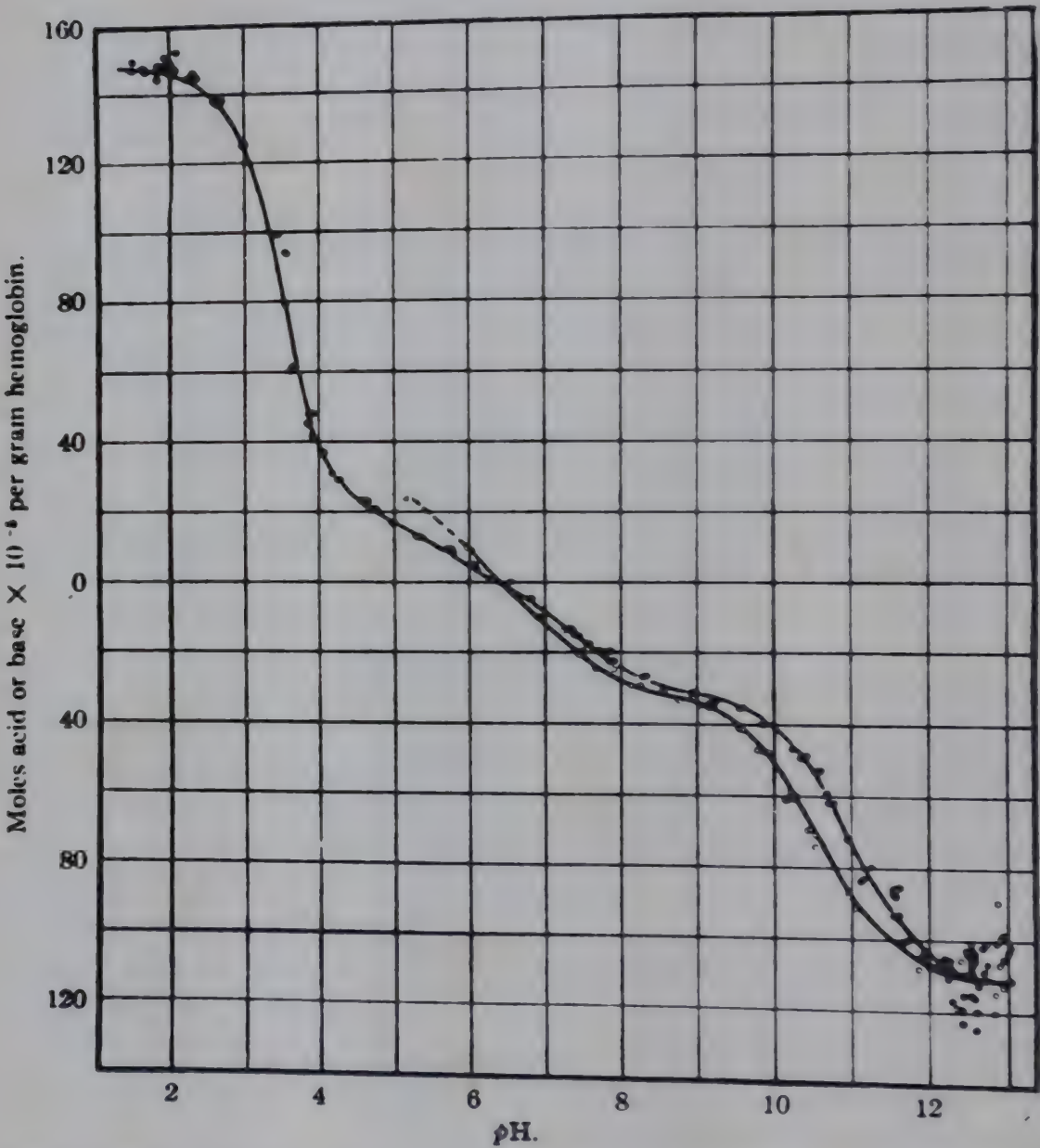


FIG. 13. The titration curve of carboxyhemoglobin of the horse in the absence of added salt ● and in the presence of 1 M NaCl ○. From Cohn, E. J., Green, A. A., and Blanchard, M. H.: *J. Am. Chem. Soc.*, 59:509 (1937).

Ionizable Groups of Amino Acid Residues	Equivalents per Mole
Carboxyl of aspartic and glutamic	87
Imidazole of histidine	33
Lysine, arginine and tyrosine	54
Total	<hr/> 174

From the magnitudes of the dissociation constants of the ionizing groups of proteins (Table IV), the dissociation of a protein may be expected to be concentrated in three zones centered about the pH values, 4, 7, and 10–12. Aid in delimiting the limits of the pH of each zone is obtained by the observed changes in the $\Delta\text{pH}/\Delta T$, values. The experimental values of the heats of ionization, obtained from the temperature coefficient of the pH, indicate that the seg-

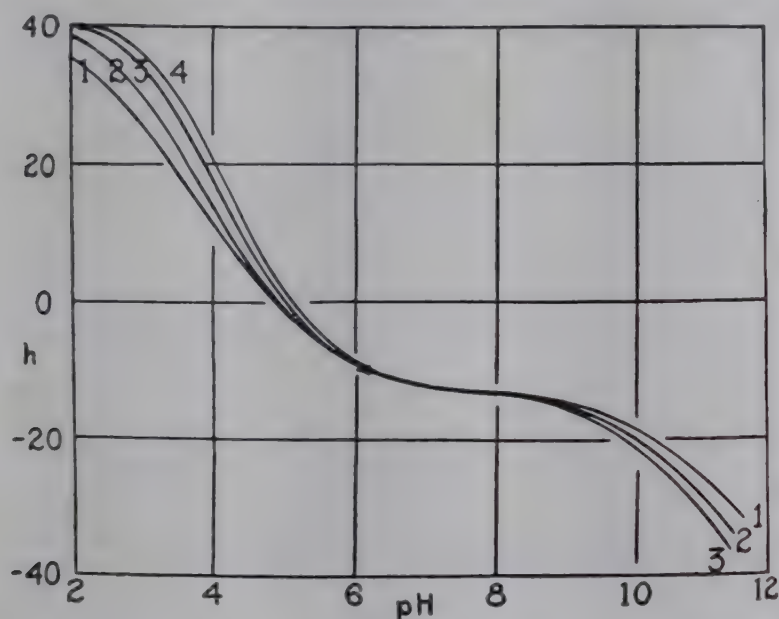


FIG. 14. Dissociation curves of crystalline egg albumin in varying concentrations of KCl; curve 1—0.033 M KCl; curve 2—0.133 M KCl; curve 3—0.667 M KCl; curve 4—2.38 M KCl. Cannan, R. K., Kibrick, A., and Palmer, A. H.: *Ann. New York Acad. Sci.*, 41:243 (1941).

Ionizable Groups	Equivalents per Mole	
	By Titration	By Analysis
(a) Total cations	41	
(b) carboxyl-guanidine	37	
(c) amino	22	15 (lysine)
(d) Imidazole	5	4
(e) guanidine = a - c - d	14	14
(f) carboxyl = b + e	51	47

ment of the curve which lies below pH 5 is probably due to the dissociation of carboxyl groups,⁹ the segment between pH 6 and 8.5 to imidazole groups, and the most alkaline branch to the ionization of amino groups. The alkaline segment might be expected to be a composite of the dissociation of amino, guanidine, phenolic and sulfhydryl groups. The guanidine groups probably occur only as cations below about pH 11. The effect of the phenolic and sulfhy-

⁹ Below pH 6 the acid groups will be the free terminal carboxyls of glutamic and aspartic acid.

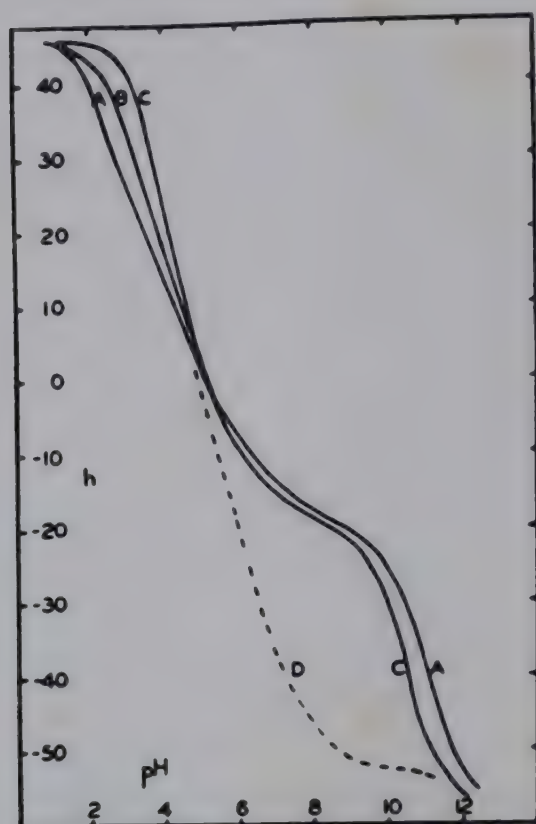


FIG. 15. Dissociation curves of β -lactoglobulin. Curve A, 0.019 *M* potassium chloride, 0.5% protein; curve B, 0.135 *M* potassium chloride, 0.5% protein; curve C, 0.67 *M* potassium chloride, 0.5% protein; curve D, 1 *M* formaldehyde, 2% protein. From Cannan, R. K.: *Chem. Revs.*, 30:395 (1942).

Ionizable Groups	Equivalents per Mole	
	By Titration	By Analysis
(a) Total cation	46	
(b) carboxyl-guanidine	52-53	
(c) carboxyl-guanidine-amino.	18-19	
(d) Imidazole	6	4
(e) Amino = b - c	34	27 (lysine)
(f) Guanidine = a - d - e	6	7
(g) Carboxyl = b + f	58	59

dryl groups remains somewhat obscure. From the listed pK values, they should dissociate between pH 9 and 11. However, titration of several proteins in the presence of formaldehyde, showed no buffering region which could be attributed to these groups. In the case of the sulfhydryl, few of the soluble proteins contain any significant amounts of this group.

4. Theory of the Protein Dissociation Curve

The dissociation curve of a polyvalent ampholyte can be simulated by that of an appropriate mixture of univalent acids (52) although the choices in the number, proportions in which they are

mixed, and the dissociation constants of the univalent acids are highly arbitrary and need not necessarily be the same as the ionizing groups in the protein. Linderstrøm-Lang (44) improved on this situation by deriving equations for the dissociation curve particularly designed to describe the effect of ionic strength on the dissociation of a protein.

The derivations of Linderstrøm-Lang's equations are based on Bjerrum's theory of electrostatic interaction in the dissociation of polyvalent acids and on the Debye-Huckel theory of the interaction of strong electrolytes. He assumed that (a) the protein may be regarded as a sphere of a given radius on the surface of which the charged groups are randomly distributed; (b) that all dissociating groups of a given kind may be described by a single intrinsic dissociation constant, K_0 ; and (c) that the dissociation of the several kinds of ionizing groups in the protein do not overlap significantly. Kirkwood (32) has extended the above derivation, eliminating the assumption that the dissociations of the several kinds of ionizing groups do not overlap. It is advantageous, however, to preserve this assumption as it allows the dissociation of each kind of group to be treated separately, like that of a symmetrical polyvalent acid. The whole dissociation curve of the protein may be obtained by combining the separate curves which will deviate only slightly from the true curve in the restricted regions in which significant overlapping occurs. In a protein the separate segments of the dissociation curve are the contributions of the carboxyl, imidazole, and amino groups. The guanidino group, being a strong base, will only affect the dissociation curve, over the pH range usually considered, by contributing a fixed cation charge to the net charge.

The derivation of the Linderstrøm-Lang dissociation equations reproduced from the publications of Cannan (8-10) is given below.

Consider a protein having m acid groups of the "A" kind. Let the protein have a fixed cation charge, n , equal to the sum of the basic groups and a variable net charge, $h = (n - x)$, the magnitude of which will depend on the number of protons, x , which have dissociated from the m acid groups. The successive dissociations of the m protons may be described by m stoichiometric constants, K_1' , K'_x , K'_m , which are related to the m thermodynamic dissociation constants as follows:

$$K'_x = \frac{P_{n-x}}{P_{n-x+1}} = K_x \frac{\gamma_{n-x+1}}{\gamma_{n-x}} \quad (XX)$$

where P represents the concentration and γ the activity coefficient

of the protein ion the charge of which is denoted by the subscript.

To combine the m equations represented by XX, let C_p be the bulk concentration of protein. Then

$$\frac{P_{n-x}}{C_p} = \frac{K_1' K_2' \cdots K_x' \alpha_H^{m-x}}{\alpha_H^m + \sum_{x=1}^m K_1' K_2' \cdots K_x' \alpha_H^{m-x}} \quad (\text{XXI})$$

and the net charge h is given by

$$h = \frac{n\alpha_H^m + \sum_{x=1}^m (n-x) K_1' K_2' \cdots K_x' \alpha_H^{m-x}}{\alpha_H^m + \sum_{x=1}^m K_1' K_2' \cdots K_x' \alpha_H^{m-x}} \quad (\text{XXII})$$

On the basis of Bjerrum's theory of the dissociation of polyvalent acids, we may define K_x in terms an intrinsic constant, K_0 , and a charge interaction quantity, $b = e^{2/2\epsilon r k T}$, in which e is the charge on the electron, ϵ is the dielectric constant, r is the distance separating the proton from the charge on the protein, k is the Boltzmann molecular gas constant, and T is the absolute temperature. The exact relation depends on whether the acid groups of the "A" kind are uncharged or are positively charged. For an uncharged acid group, e.g., COOH , we have:

$$K_x' = K_0 \cdot e^{2b(n-x+1)} \cdot (m-x+1)/x \quad (\text{XXIII})$$

and for a positively charged acid, e.g., NH_3^+ ,

$$K_x = K_0 \cdot e^{2b(n-x)} \cdot (m-x+1)/x. \quad (\text{XXIV})$$

The Debye-Hückel expression for the activity coefficient of a polyvalent ion is now introduced to provide a relation between K_x' and K_x . This gives

$$-\ln \gamma_1 = \frac{e^2}{2\epsilon k T} \frac{\kappa}{1 + \kappa a} \quad (\text{XXV})$$

$$\gamma_{n-x} = e^{-(n-x)^2 \ln \gamma_1} \quad \text{and} \quad \frac{\gamma_{n-x+1}}{\gamma_{n-x}} = e^{2(n-x+0.5) \ln \gamma_1} \quad (\text{XXVI})$$

κ being the thickness of the ion atmosphere which is a function of the ionic strength, and a the distance of closest approach of the ions of the atmosphere to the protein ion. Introducing equation XXVI into equation XX and combining with equations XXIII

and XXIV, we have

$$K_x' = K_0^0 \cdot e^{2w(n-x+0.5) \cdot (m-x+1)/x} \quad (\text{XXVII})$$

which, on conversion to common logarithms, becomes

$$pK_x' = pK_0^0 - 0.868w(n-x+0.5) - \log \frac{m-x+1}{x} \quad (\text{XXVIIa})$$

In equations XXVII and XXVIIa

$$K_0^0 = K_0 e^{\pm b}$$

and

$$w = b + \ln \gamma_1 = b \left[1 - \frac{\kappa r}{1 + \kappa a} \right] \quad (\text{XXVIII})$$

The sign of the exponent **b** in equation XXVIII is *plus* for the case of an uncharged acid group, and *minus* for the case of a positively charged acid group. The quantity **w** is a function relating the charge interaction, **b**, to **r**, the distance between the proton and the charge on the protein.

To apply equation XXVIII numerically, the dielectric constant (ϵ) of the protein solution is assumed to be the same as that of the solvent. Then, at 25°, $\epsilon = 78.8$ and $b = 3.538 \times 10^{-8}/r$. Applying the assumptions that the protein is a spherical molecule and that there is a random distribution of the electric charge, **r** may be considered to be the radius of the protein molecule. Then $a = r + r'$, where r' is the effective mean radius of the ions surrounding the protein. In solutions of potassium chloride, r' may be assigned the value of 2Å (45). For nearly all proteins **r** may be expected to exceed 20Å. Therefore, the value of **b** should not exceed 0.17 and that of **w** should vary with $\mu = 0$ to $\mu = \infty$ only between the limits of 0.17 and 0.

For fixed values of **m**, **n** and K_0^0 , any selected value of **w** may be introduced into equation XXVIIa to yield the **m** dissociation constants. With the aid of the dissociation constants (K_0^0), **h** values for the construction of a dissociation curve may be computed from equation XXII.

Equations XXVIIa and XXII are applied to an experimental curve in the following manner: (a) The values of **m** and **n** are obtained from the stoichiometric analysis of the curve. (b) If $m > n$, as is the case with many proteins, the isoionic point (**pI'**) will fall within the carboxyl section of the curve. At the isoionic point, $h = 0$, and a value for K_0^0 may be derived from the equation

$$\alpha_H = K_0 \frac{m - (n - h)}{n - h} \quad (\text{XXIX})$$

and the experimental value of pI' . Equation XXII has been shown to reduce to XXIX in the absence of interaction between the ampholyte ion and the proton or the ion atmosphere (8). The m constants defined by equation XXVIIa are symmetrically distributed about their mean, which is identical with $pK'_{m+1/2}$ and with the mid-point of the dissociation curve ($pH_{mid.}$). It follows that the dissociation curve is also symmetrical about its mid-point and as a consequence

$$pH_{mid.} = pK'_{m+1/2} = pK_0^0 - 0.868w \frac{2n - m}{2} \quad (\text{XXX})$$

With K_0^0 being known, w may be computed from the experimental value of $pH_{mid.}$ by equation XXX.

The theoretical dissociation curve is approximately linear in the region of the mid-point and Linderström-Lang has shown that the slope on the basis of this assumption is

$$\frac{\Delta pH}{\Delta h} = -0.868 (w + 2/m). \quad (\text{XXXI})$$

From equation XXXI a value of w may be obtained graphically without knowledge of K_0^0 and, conversely, this constant can then be computed with the aid of equation XXX.

By the above methods, all quantities necessary for the solution of equations XXII and XXX are made available, and a comparison

TABLE V
VALUES OF K_0^0 OF β -LACTOGLOBULIN (10)

Carboxyl Groups; $m = 58$; $n = 46$					Imidazole Groups; $m = 6$; $n = -12$	
μ	$-\frac{\Delta pH}{\Delta h}$	$w_{exp.}$ (Equation XXXI)	$pH_{mid.}$	pK_0^0 (Equation XXX)	$pH_{mid.}$	pK_0^0 (Equation XXX)
0.010	0.083	0.060	3.70	4.60	7.55	6.76
0.019	0.075	0.052	3.82	4.59	7.45	6.78
0.035	0.070	0.046	3.92	4.60	7.35	6.75
0.069	0.063	0.038	4.03	4.59	7.28	6.78
0.135	0.058	0.032	4.10	4.58	7.23	6.82
0.270	0.0535	0.027	4.19	4.59	7.16	6.82
0.670	0.0475	0.020	4.30	4.60	7.10	6.83
2.10	0.043	0.015	4.38	4.60	7.00	6.80
$pI' = 5.18$ (Equation XXIX)					4.60	

TABLE VI

COMPARISON OF CALCULATED AND OBSERVED VALUES OF ACID-BASE COMBINING CAPACITY OF β -LACTOGLOBULIN (10)Theoretical Curve for $w = 0.038$ ($\mu = 0.069$) (Equations XXVIIa and XXII)

pH	h	
	Theory	Observed
1.5	44.7	
2.0	42.6	42.4
2.5	38.6	38.4
3.0	32.7	32.5
3.5	25.4	25.5
4.0	17.6	17.5
4.5	9.7	9.6
5.0	2.3	2.3
5.5	- 3.8	- 3.5
6.0	- 8.1	- 7.8
6.5	-10.5	-10.8

 $pK_0^0 = 4.60$; $m = 58$; $n = 46$.

can be made of the experimental curve with the theoretical curve which corresponds with these experimental values of K_0^0 , w , m , and n . This procedure has been applied to the curves of egg albumin and of β -lactoglobulin. The results are reproduced in Tables V to VII. Only the carboxyl regions of the curves are sufficiently broad to permit a full analysis. It has been found that a single value of w does suffice to describe practically the whole span of dissociation of the carboxyl groups at constant μ . Moreover, w has been found to vary with μ in a manner qualitatively consistent with the theory. However, the values of w which best described the curves were all somewhat smaller than the values calculated from equation XXVI, employing $\epsilon = 78$ and computing r from the diffusion constant of the protein. The ratio of w (theory) to w (found) was about 1.25 for egg albumin and between 1.05 and 1.1 for β -lactoglobulin. So many dubious assumptions are involved in the calculation of w that larger discrepancies than those found might well have been anticipated.

The values of w which were found to describe the dissociation of the carboxyl groups were used to calculate theoretical curves for the imidazole and amino groups of the two proteins. In the case of egg albumin they did fit the experimental curves quite satisfactorily. The theory, however, failed to account for the slopes and positions of the amino segments of the curves of β -lactoglobulin. The values of pK_0 which were found to apply to egg albumin were 4.2

TABLE VII
THEORETICAL DISSOCIATION CURVES OF CRYSTALLINE EGG
ALBUMIN (9)

μ	0	0.067	∞
w	0.128	0.043	0.00
0.7w	0.09	0.03	0.00
h		pH	
38		1.96	3.09
36	0.10	2.25	3.33
34	0.41	2.48	3.49
32	0.72	2.68	3.62
30	1.05	2.85	3.73
28	1.30	3.01	3.82
26	1.58	3.14	3.91
24	1.80	3.27	3.99
22	2.08	3.40	4.06
20	2.33	3.52	4.13
15	2.94	3.86	4.31
10	3.58	4.18	4.48
8	3.83	4.31	4.55
6	4.07	4.45	4.63
4	4.34	4.58	4.71
2	4.61	4.74	4.80
0	4.90	4.90	4.90
-2	5.20	5.08	5.02
-4	5.52	5.29	5.16
-6	5.88	5.52	5.35
-8	6.32	5.85	5.60
-10	6.90	6.32	6.03
-12	7.66	6.95	6.60
-14	(8.6)	(7.8)	(7.3)
-16	(10.1)	(9.2)	(8.7)
-18	10.85	9.77	9.25
-20	11.30	10.10	9.52
-22	11.68	10.38	9.71
-24	12.04	10.61	9.88
-26	—	10.82	10.03
-28	—	11.02	10.18
-30	—	11.25	10.34

(carboxyl), 6.7 (imidazole), and 10.0 (amino), while those of β -lactoglobulin were 4.5 (carboxyl) and 6.7 (imidazole).

5. An Approximate Dissociation Equation

The computations involved in the solution of equation XXII are so tedious that a simpler relation was sought which would approximate this equation for a representative range of values of w and m . Now, when $w=0$, the curve obtained is identical with that of m equivalents of a univalent acid for which $K=K_0^0$. That is to say, if α represents the fraction of the m groups which have dissociated a proton, then

$$\text{pH} = \text{p}K_0^0 - \log (1 - \alpha) / \alpha. \quad (\text{XXXII})$$

A series of curves for a range of positive values of w and a fixed value of m were then constructed from equation XXII. When these were compared with the curve for equation XXX, it was found that they were displaced from it by an amount approximately equal to $0.868wh$. That is to say,

$$\text{pH} = \text{pK}_0^0 - \log \frac{(1-\alpha)}{\alpha} - 0.868wh. \quad (\text{XXXIII})$$

That this relation would hold in the linear segments of the curve is implicit in equation XXX. It was encouraging to find that it applied with fair precision over the whole course of a family of curves corresponding to a range of values of w wider than that to be expected in proteins.

At the mid-point ($\text{pH}_{\text{mid.}}$) of the curves $\alpha = 0.5$. Consequently, since $\alpha = (n-h)/m$,

$$\text{pH}_{\text{mid.}} = \text{pK}_0^0 - 0.858w(n-0.5m)$$

and

$$\text{pH} = \text{pH}_{\text{mid.}} - \log (1-\alpha)/\alpha - 0.868wm(\alpha-0.5). \quad (\text{XXXIV})$$

According to equation XXXIV, the slope of the curve relating pH to α should be uniquely determined by the value of the product $w m$. This offers a simple means of exploring the magnitude of the deviations of the approximate equation XXXIII from equation XXII.

The dissociation equations deduced in this section fitted the titration data of carboxyhemoglobin very poorly (22). The investigators assume that the disagreement was due to overlapping of interaction between the dissociating groups.

X. TITRATION THROUGH FORMATION OF INSOLUBLE PROTEIN SALTS

1. Dye-Protein Titrations

The uncertainties in estimating the acid- and base-combining capacities of proteins from their titration curves has previously been pointed out. The difficulty of determining the small differences between the total and free acid or base added and the problem of estimating the activity coefficients of the hydrogen and hydroxyl ions can be eliminated by employing acids and bases that form insoluble stoichiometric compounds with proteins. The use of dyes for determining the acid- and base-combining capacities of

proteins was studied by Chapman, Greenberg and Schmidt (11) and has more recently been extended by Fraenkel-Conrat and Cooper (18). The essence of the method is to add an excess of acidic or basic dye to a protein at sufficient acidity or alkalinity so that all the cationic or anionic groups of the protein will be neutralized by the acidic or basic dye respectively, yielding a precipitate that can be removed. The excess of dye remaining in the solution is determined and the difference gives the amount that has reacted with the protein.

The basis of the dye-protein titration can be illustrated by means of the simplified considerations used as a model that are given below.

Assuming that the reaction between dye and protein obeys the solubility product principle, then

$$P^+ \cdot D^- = K_s$$

where K_s is the solubility constant. The dye is considered to be essentially completely ionized so that $D^- = D$, the total dye concentration. The dissociation of the protein cation is given by equation VIIa. If the total protein concentration is represented by the letter P , then

$$P^+ = \frac{\alpha_H \cdot P}{K_1 + \alpha_H} .$$

Combining these two equations gives

$$K_s = \frac{\alpha_H P \cdot D}{K_1 + \alpha_H} .$$

The corresponding equation for the reaction between a protein anion and a basic dye cation is

$$K_s = \frac{K_2 P \cdot D}{K_2 + \alpha_H} .$$

In the titration of proteins with strong acid dyes, it will be noted from Table IV that the strongest acid group contributing to the dissociation curve of a protein is the carboxyl group with a mean pK value of about 4. It becomes obvious then that the precipitation of the dye-protein compound will be little affected by the pH of the solution at pH values below 3.

In the titration of proteins with basic dyes, the reaction cannot

be carried out in a sufficiently alkaline region where the pH does not have a significant influence because of the strong basicity of the guanidine group ($pK = 12.5$). However, if the product of the concentrations of protein and dye is increased sufficiently, it can offset the effect of incomplete dissociation. As an example, consider that the guanidine group is to be titrated, $K = 10^{-12.5}$, and the titration is carried out at a value of $\alpha_H = 10^{-11}$ ($pH = 11$). Introducing these terms into the above equation it may be seen that the product of $D \times P$ will have to be between 10- to 100-fold greater than the value of the constant, K_s . This condition seems to be met as is shown by the fact that the dye titration curves of Rawlins and Schmidt (60) approach a constant limiting value. This is also confirmed by the experiments of Fraenkel-Conrat and Cooper (18).

Refinement in the technique of dye titrations through the use of buffered acid or alkaline solutions and the photoelectric determination of the uncombined dye has been made by Fraenkel-Conrat and Cooper (18). The basic groups in proteins are determined by these investigators by titrating with the sulfonic acid dye, orange-G, in the presence of a citrate buffer of $pH\ 2.2$.¹⁰ The analysis is carried out by adding increasing amounts of 0.1% orange-G solution to 5 mg. samples of the protein in a series of 15 ml-test tubes. Several glass beads are added, and the tubes are shaken in a mechanical shaker for 20 to 24 hours. The tubes are then centrifuged, the supernatant solution collected and diluted 100-fold and the color intensity read in a photoelectric colorimeter, using a blue filter. The dye concentration is determined from a standard calibration curve prepared by diluting an orange-G stock solution. The dye bound by protein is obtained by subtracting the excess found in the supernatant from the amount added. Orange-G has a molecular weight of 452 and is a dibasic acid.

The acidic groups in a protein are similarly determined with safranin-O, employing a buffer of $pH\ 11.5$ ¹¹ and 0.2% safranin solution. Calculation of the equivalent values of the acid groups with this dye is somewhat ambiguous since safranin-O is a mixture of homologous monoacid bases; the molecular weights of the two main constituents being 350.5 and 364.5. Fraenkel-Conrat and Cooper based their calculations on the molecular weight mean of 355.

Saturation of the protein with either acid or basic dye is indicated

¹⁰ Prepared by mixing 980 ml. of 0.1 *M* citric acid with 20 ml. 0.2 *M* Na_2HPO_4 .

¹¹ Prepared by mixing 250 ml. of 0.2 *M* Na_2HPO_4 and 200 ml. of 0.1 *M* NaOH and adding water to 1 liter.

TABLE VIII
COMPARISON OF BASIC GROUPS OF PROTEINS AS DETERMINED
BY VARIOUS METHODS (18)

Protein ¹	Basic Residues per Gm. Protein $\times 10^4$			
	Dye-Binding Capacity ²	Titration	Metaphosphoric Acid-Binding Capacity	Analysis or Isolation
Egg albumin	8.8 ³	8.0– 8.7	7.8– 8.0	9.0
β -Lactoglobulin	11.6 ³	11.5		11.5
Casein	6.8	7.6– 9.0		8.0–9.4 ⁴
Fibrin	12.0			13.1 ⁴
Gelatin	6.0	8.9– 9.6	10.3–11.0	10.7 ⁴
Gliadin	4.3 ³	3.4		4.4 ⁴
Insulin	9.4 ³	10.1		9.5
Lysozyme	11.5 ³	11.7–12.8		12.2
Zein	1.9	1.8– 2.1		1.5

¹ Egg albumin and β -lactoglobulin were electrodyalyzed; all proteins were corrected for moisture content. Casein, gelatin, gliadin, and zein were commercial preparations. Two casein preparations gave identical values.

² Moles of orange-G bound at pH 2.2 multiplied by 2.

³ These analyses represent averages of data obtained with 5 and 1.5 mg. protein samples, with protein concentrations ranging from 0.1 to 0.2 and from 0.06 to 0.15 percent, respectively. Results of the two techniques agreed within 5 percent.

⁴ Determined by nitrogen distribution.

by lack of a progressive trend in the amount of dye bound as the dye concentration is increased.

The results of dye titrations on a number of proteins are given in Tables VIII and IX.

2. Metaphosphoric Acid Titration

Addition of excess of metaphosphoric acid leads to the formation of a protein precipitate in which the protein and precipitant appear

TABLE IX
COMPARISONS OF TOTAL ACID AND CARBOXYL GROUPS OF PRO-
TEINS WITH THEIR CAPACITY TO BIND SAFRANINE (18)

Protein ¹	Dye Bound ²	Total Acid Groups ³	Carboxyl Groups ³
Egg albumin	13.5 ⁴	13.8	10.4
β -Lactoglobulin	17.6 ⁴	17.5	14.5
Casein	19.4	16	13
Gelatin	12.7	10	10
Gliadin	5.5	7	5
Insulin	17.5	13	6
Zein	5.5	6	3

¹ See the corresponding footnote to Table VIII.

² Moles of safranine-O bound at pH 11.5 by 10^4 gm. of protein (range of protein concentrations, 0.1 to 0.2 percent).

³ Of 10^4 gm. of protein; calculated from analyses for glutamic and aspartic acids, amide N, tyrosine, and cysteine, and as amended for glutamic acid of insulin, gelatin, and gliadin. The unknown number of terminal carboxyl groups of the polypeptide chains was disregarded.

⁴ In more dilute solution (protein concentrations 0.06 to 0.15 percent), 12.4 and 16.5 moles of dye were bound by egg albumin and β -lactoglobulin, respectively.

to be combined in stoichiometric proportions. The uniformity of this relationship suggests that precipitation by metaphosphoric acid may be used to determine the acid-combining capacity of proteins (55). The analysis is carried out by the addition of an amount of 0.1*N* metaphosphoric acid to 5 ml of a protein solution containing 10.0 to 15.0 mg. of protein nitrogen sufficient to lower the pH to less than 2.0. The protein precipitated is isolated by centrifugation and washed until the washings contain neither protein nor phosphorus. Each precipitate is then dissolved in concentrated sulfuric acid and analyzed for its nitrogen and phosphorus content. Representative values are given in Table VIII.

Similar stoichiometric relationships in the precipitation of proteins have been obtained with alkaloids (56) and complex metal salts (47). Michael (47) observed that the anionic complex salts, e.g., $K_3[Cr(CNS)_6]$, $K[Cr(CNS)_4(NH_3)_2]$, $K_3[Co(CN)_6]$ —precipitate proteins in solutions more acid than the isoelectric point, whereas cationic complex salts—e.g. $[Cr(NH_3)_6]Cl_3$, $[Co(NH_3)_4CO_3]Cl$, $[Cr(CO(NH_2)_2)_6]Cl_3$ —precipitate on the alkaline side of the isoelectric point without any accompanying denaturation. The amount of complex anions combined in the precipitates represents the maximal acid-combining capacity of the protein; the amount of complex cation, at least in neutral solution, only a fraction of the maximal base-combining capacity.

XI. METALLIC COMPLEXES OF PROTEINS AND AMINO ACIDS

1. Structure

Abundant evidence is available to show that proteins, and also the amino acids, form complexes with many of the metallic cations. In the case of the proteins it seems probable that, with the exception of the alkali elements, some degree of complex ion formation occurs on the alkaline side of the isoelectric point with all the metals.

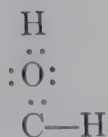
A metallic complex is here considered to be a chemical compound formed between the cation and an anion (organic or inorganic) in which the activity of the metallic ion is greatly decreased, and in which the electrical charge carried by the compound may be equal to, but, generally is different from that of the constituent cation either in sign or in magnitude.

A satisfactory structural explanation has been developed for the stability of the complexes of certain of the elements; for others it is

still in an uncertain state. Pauling (54) points out that the elements of the transition series (iron, copper, and zinc groups of the periodic system) can form multiple covalent bonds with electron-accepting groups by making use of the electrons and orbitals of the shell within the valence shell. This confers a large amount of double-bond character upon the covalent bonds of these elements. Other factors that have to be considered are the electronegativity of the attached groups, the acid character of the element, and the formation of chelate rings.

In the case of the binding of many of the metallic cations, particularly of the alkali earth elements, there is no ready structural explanation of complex formation. The prevention or the retardation of the ionization of the alkali earth cations from their compounds with the more non-metallic elements must be mainly due to electrostatic forces (20).

To explain complex formation with cations of the above type requires the demonstration of the action of some other force in addition to the usual ionic bond between an anion and a cation. Smythe and Schmidt (65) pointed out that this additional force may be derived from the amount of residual negative charge on certain groups ($-\text{OH}$, $-\text{COOH}$, $-\text{NH}_2$) of amino acids and proteins due to the attractive forces of unequal nuclear charges. This may be calculated approximately from the rule of Latimer and Porter (35) that when a pair of electrons is shared between two atoms, except in the case of hydrogen, the effect of their charges is distributed between the atoms in the ratio of the positive charges on the two nuclei. Thus a pair of electrons between carbon and oxygen contributes 0.4×2 electron charges to the carbon atom and 0.6×2 electron charges to the oxygen. As an example we may consider the charge on a hydroxyl group which has the electronic arrangement,



The nuclear charge of oxygen is +6, of carbon +4. The 2 electrons between these atoms, it is assumed, will be shared in this ratio. The unshared electrons will react completely toward neutralizing the nuclear charge on the oxygen, and hydrogen is considered to be sufficiently close to the center of the negative charge between it and oxygen to just neutralize one electron. Summing up the posi-

tive and negative charges there is obtained,

$$6 - 4 - (2 \times 1/2) - (2 \times 0.6) = -0.2.$$

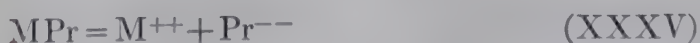
Therefore, according to this calculation, the oxygen of the hydroxyl group possesses a residual negative charge of 0.2 electron unit. In a similar manner one may calculate that the residual charge on the carbonyl oxygen of a carboxyl group is -0.4 electron unit. The relatively high dielectric constants exhibited by alcohols afford a physical demonstration of the considerable negative charge on the hydroxyl group.

The spatial distribution of the atoms in a compound as well as the force involved is an important factor in determining whether a complex will be formed. Among the most important of the spatial arrangements are those which favor ring closure. Metal ions which are good electron acceptors, namely the iron, copper, and zinc groups of the periodic table, readily complete ring closure forming chelate rings (14, 58). In these chelate rings, the metal shares a pair of electrons with each of two atoms in such a manner as to complete the ring. The metal ions are held in the ring by covalent bonds. Compounds with reactive atoms spaced so as to permit formation of five or six membered rings, chelate most readily, since these are the rings of the greatest stability.

2. Application of the Law of Mass Action

Most of the evidence for the formation of metal-protein and metal-amino acid complex ions is of a qualitative nature. Quantitative information on the stoichiometry, the dissociation mechanism, or the dissociation constants of the complexes is scant. However, in certain instances, particularly in the reaction of the alkali earth cations with proteins it has been possible to apply the law of mass action to a first approximation (20).

The dissociation of a metal proteinate may be written for the present purpose as



Formulating in terms of the mass law gives

$$\frac{[M^{++}][Pr^{--}]}{[MPr]} = K_{MPr} \quad (XXXVI)$$

where [] represents molar concentrations.

It is not possible to make an independent determination of the protein ion concentration. Since $[TPr] = [MPr] + [Pr^{--}]$, where

TABLE X
DISSOCIATION CONSTANTS OF CALCIUM PROTEINATES (20)

Method	Solution	pH	Temperature	Conversion Factor	pK
CASEIN					
Frog heart	NaOH-H ₂ O	7.35	22°	0.300	2.38 ± 0.02
CaCO ₃ solubility	Modified Ringers (μ = 0.160)	7.2-7.7	38°	0.293-0.355	2.23
Ultracentrifugal analysis	NaOH-H ₂ O	6.3-8.5	5°	0.40	2.73
Ultracentrifugal analysis	NaCl-NaOH-H ₂ O	6.3-8.5	5°	0.40	2.36
TOTAL SERUM PROTEIN					
Frog heart	Serum and body fluids	—	22°	0.122	2.22 ± 0.07
Frog heart	Serum	—	—	—	2.17 ± 0.06
Ultrafiltration	Serum	—	25°	0.062	2.44
SERUM PROTEINS					
(a) Serum globulins					
CaCO ₃ solubility	Modified Ringers μ = 0.160	7.0-7.9	38°	0.081-0.119	2.36
Frog heart	Modified Ringers μ = 0.160	7.4	22°	0.05	2.96 ± 0.13 ¹
Frog heart	Modified Ringers μ = 0.160	7.4	22°	0.11	2.70 ± 0.14 ²
Frog heart	Modified Ringers μ = 0.160	7.4	22°	0.055	2.00 ± 0.10 ³
Frog heart	Modified Ringers μ = 0.160	7.4	22°	0.145	3.13 ± 0.14 ⁴
(b) Total serum albumin					
CaCO ₃ solubility	Modified Ringers μ = 0.160	7.3-7.8	38°	0.139	2.11

¹ Constant for pseudoglobulin—from normal horse serum.
² Constant for euglobulin P_I.
³ Constant for euglobulin P_{II}.
⁴ Constant for euglobulin P_{III}.

TPr represents the total protein, on substitution for [Pr⁻⁻] there is obtained

$$\frac{[M^{++}] \cdot ([TPr] - [MPr])}{[MPr]} = K_{MPr}. \tag{XXXVII}$$

Rearranging gives

$$\frac{[TPr]}{[MPr]} = 1 + \frac{K_{MPr}}{[M^{++}]}. \tag{XXXVIII}$$

To make numerical use of equation XXXVIII requires the assumption that

$$[\text{TPr}] = f \cdot \text{TPr} \quad (\text{XXXIX})$$

where f is a factor for converting protein concentration from the usual units of gram per 100 ml. or per liter of water to molar units when the metallic ion combined with the protein has its maximum value; namely when $M^{++} = \infty$.

When equation XXXIX is introduced into equation XXXVIII there is obtained

$$\frac{\text{TPr}}{[\text{MPr}]} = \frac{1}{f} \left(1 + \frac{K_{\text{MPr}}}{[M^{++}]} \right). \quad (\text{XL})$$

To apply equation XL, it is necessary to be able to evaluate the conversion factor f . This may be done from the experimental data by plotting $\text{TPr}/[\text{MPr}]$ against $1/[M^{++}]$. This yields a straight line if equation XL is valid. Extrapolation to the value of $1/[M^{++}] = 0$ gives the factor f as the intercept on the $\text{TPr}/[\text{MPr}]$ axis.

Values for the dissociation constants of calcium proteinates are given in Table X.

It is obvious that the protein molecule can combine with more

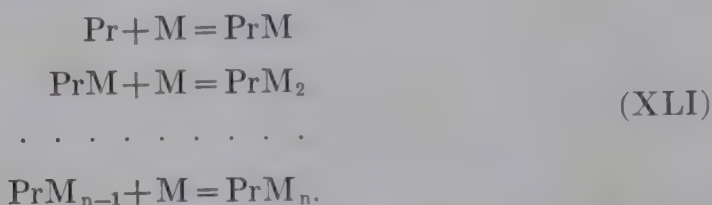
TABLE XI

SUMMARY OF COPPER COMPLEXES OF GLYCINE AND ALANINE (5)

Complex	Range of Stability	Probable Formula of Complex	H Ions Set Free per Atom Cu Bound	Absorption Characteristics
1st acid glycine	about pH 0.5-2.5.	Cu glycine ₂	1	Curve similar to Cu-(OAc) ₂ in alcohol.
2nd acid glycine	pH 2-5; in high dilution, up to pH 7.	Cu ₂ glycine ₃	0.5	Peak just in infra red.
Neutral glycine	pH 5-8; overlapping basic complex to pH 10.5.	Cu glycine ₂	1	Peak at 6250 Å.
Basic glycine	pH 8-12; not affected by dilution.	Cu glycine ₂	2	Peak at 6700 Å.
1st acid alanine	about pH 0.5-2.5.	Cu alanine ₂	0	Very low absorption; about half that of 1st acid glycine and close to that of cupric ion.
2nd acid alanine	pH 2.5-6; favored by dilution.	Cu ₂ alanine ₃	0.5	Exactly similar to 2nd acid glycine.
Neutral alanine	pH 5-9; in dilute solutions to pH 11.	Cu alanine ₃ or Cu ₂ alanine ₅	1	Peak at 6200 Å.; higher than neutral glycine.
Basic alanine	pH 8-11; only in concentrated solutions.	Cu alanine ₅	2	Peak at 6460 Å.; higher than basic glycine.

than one metallic cation and equation XXXVIII can be only an approximate representation of the true situation. Klotz (34) has made a more rigorous application of the law of mass action and has found the conditions under which the general treatment can be reduced to equation (XL). In brief, the general analysis shows that if one may neglect electrostatic interactions between successively bound metal ions, the equations for multiple-step binding may be reduced formally to a single-step equation but one in which equivalents rather than moles of protein is used.

The argument of Klotz is given below. The combination of the metallic ion may be represented by the steps



These reactions may be represented by the classical equilibrium equations

$$\begin{aligned} \frac{[\text{PrM}]}{[\text{Pr}][\text{M}]} &= K_1 \\ \frac{[\text{PrM}_2]}{[\text{PrM}][\text{M}]} &= K_2 \\ &\dots \dots \dots \\ \frac{[\text{PrM}_n]}{[\text{PrM}_{n-1}][\text{M}]} &= K_n \end{aligned} \tag{XLII}$$

From equation XLII there can be derived the convenient relations

$$\begin{aligned} \frac{[\text{PrM}_2]}{[\text{Pr}][\text{M}]^2} &= K_1 K_2 \\ &\dots \dots \dots \\ \frac{[\text{PrM}_n]}{[\text{Pr}][\text{M}]^n} &= K_1 K_2 \dots K_n \end{aligned} \tag{XLIII}$$

The extent of binding of metal ion by protein may be expressed by a term r , which represents the ratio of bound ions to the total moles of protein. From the quantities given in equations XLII and XLIII, r becomes

$$r = \frac{[\text{PrM}] + 2[\text{PrM}_2] + \dots + n[\text{PrM}_n]}{[\text{Pr}] + [\text{PrM}] + [\text{PrM}_2] + \dots + [\text{PrM}_n]}. \tag{XLIV}$$

By suitable substitution this equation can be transformed to

$$r = \frac{K_1[M] + 2K_1K_2[M]^2 + \dots + n(K_1K_2 \dots K_n)[M]^n}{1 + K_1[M] + K_1K_2[M]^2 + \dots + (K_1K_2 \dots K_n)[M]^n} \quad (\text{XLV})$$

Inspection of XLV reveals that if $[M]$ is factored out of the numerator, the remaining factor is the derivative of the denominator with respect to $[M]$. If the denominator is represented by f and f' represents the derivative ($df/d[M]$), equation XLV reduces to

$$r = [M]f'/f. \quad (\text{XLVI})$$

Employing this generalized notation and equation XXXVIII leads to the empirical relation,

$$r = \frac{m[M]}{K_{MPr} + [M]} \quad (\text{XLVII})$$

Now by equating the relations given in equation XLVI and XLVII, there are determined the conditions under which the law of mass action reduces to the simple expression given in XXXVI, which is

$$\frac{f'}{f} = \frac{[M]}{K_{MPr} + [M]} \quad (\text{XLVIII})$$

This is a differential equation which on integration gives

$$f = \left(1 + \frac{[M]}{K_{MPr}}\right)^m \quad (\text{XLIX})$$

or

$$1 + K_1[M] + K_1K_2[M]^2 + \dots + (K_1 \dots K_m)[M]^m = \left(1 + \frac{[M]}{K_{MPr}}\right)^m \quad (\text{XLIXa})$$

It can be shown that equation XLIXa is satisfied if the successive equilibrium constants obey the relation

$$K_n = \frac{m - (n - 1)}{n} \frac{1}{K_{MPr}} \quad (\text{L})$$

where m represents the maximum number of metal ions bound by one molecule of protein. Equation (L) may be used also to calculate the true mass law constants from the empirical intrinsic constant, K_{MPr} . From a molecular point of view it is of interest to note that equation L is the relation obtained in any system of multiple equi-

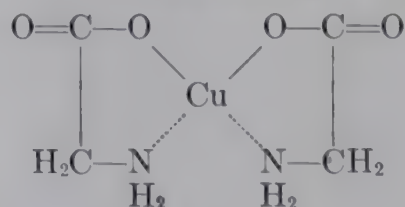
libria where the intrinsic binding affinity of the central molecule for each added ion is the same and where electrostatic interactions between successively bound ions may be neglected (34a).

3. Chelated Complexes of Amino Acids

There is an extensive literature on the metallic complexes of amino acids and proteins, but it is mostly of a qualitative nature. Quantitative determination of the composition of metallo-amino acid complexes have been attempted from measurement of absorption spectra, oxidative behavior, and activity measurements with various metal-ion electrodes. The results are not subject to easy interpretation because mixtures of compounds occur which vary with the pH and the composition of the solutions. An example is the work of Borsook and Thimann (5) on the copper complexes of glycine and alanine derived from measurements of the absorption spectra and copper ion activity determined by means of a copper electrode. A summary of the compounds deduced to be present under varying conditions is given in Table XI.

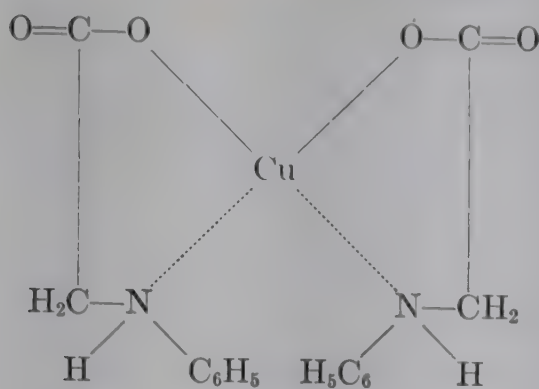
The most plausible interpretation of the structure of the metallo-amino acid complexes is that they are chelated ring compounds. The best available information is on the compounds with copper, cobalt, chromium, lead, nickel, and iron.

The monoaminomonocarboxylic acids form bidentate compounds according to the terminology of Diehls classification (14). This is exemplified by the copper complex of glycine (41) which was early shown to be only slightly dissociated by freezing point and electrical conductivity determination. The color is practically identical with that of the copper ammoniates. Apparently both the carboxyl and amino group are linked to the copper. This is explained by the structural formula having a chelate ring given below.



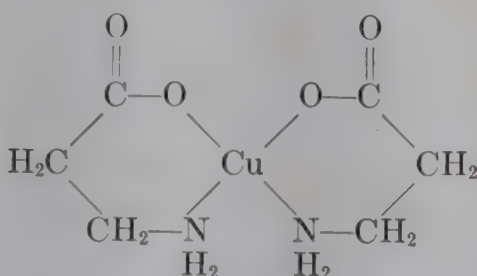
Copper glycinate

Aliphatic or aryl group substitutions on the methylene group cause little change in the color of the metallic compounds. An aryl substitution on the amino group brings about a great change in color (14). As an example, the copper compound of phenyl substituted glycine is grass green. The formula is shown below.

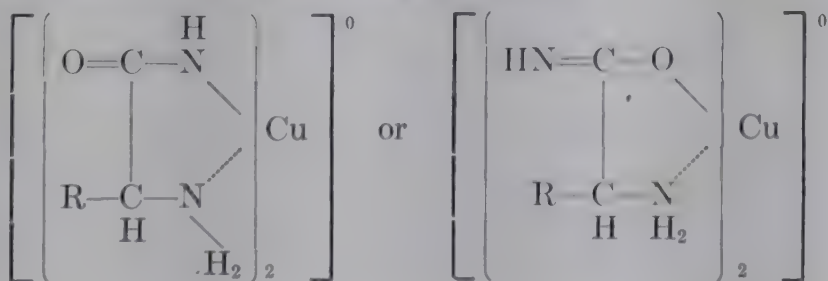


Copper phenylaminoacetate

The β -amino acids can react similarly with metals, forming a six membered ring. However, when the amino group is further removed, chelation does not occur (14). The ring structure of β -alanine would be


 Copper- β -alaninate

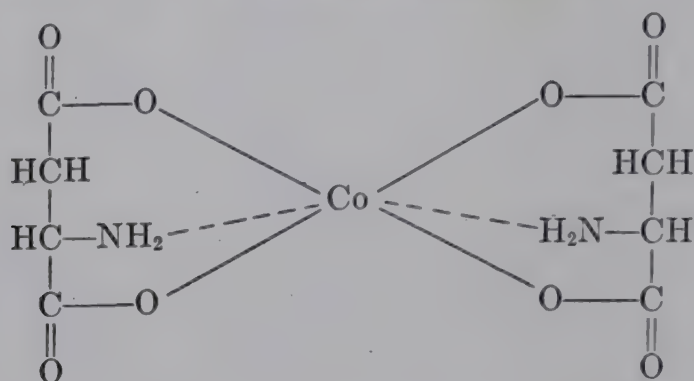
The cobalt and chromium compounds of the amino acids occur in two isomeric modifications (42) corresponding to a cis-cis and cis-trans configuration about the octahedrally arranged hexacovalent metals. Of significance for complex formation with proteins, is the observation of Rising (61) that amino acid amides yield compounds with copper, obtained in the dry states as red amorphorous powders, that are non-conducting in solution and probably have one of the inner complex structures given below.



Copper amino acid amides

The dicarboxylic amino acids, aspartic and glutamic acids, form what Diehl calls tridentate compounds. In these the two carboxyl groups are linked through primary valences and the amino group

is coordinately linked. An example is the trivalent cobalt compound of aspartic acid (43) which is deep purple in color and very soluble in water. Electrical migration indicates that the colored ion carries a negative charge (14). The assigned structure is



Cobaltic aspartate

Other probable examples of tridentate amino acid molecules are the copper derivative of aspartic acid (59) and the copper (59), lead (2) and cobalt (33) derivatives of glutamic acid.

The sulfur containing amino acids (methionine, cystine, cysteine) offer further possibilities for chelation, Diehl (14) gives examples of thiol ether compounds in which the hydrogen atom of the SH group is replaced by a metal.

The cobalt, nickel and iron complexes of cysteine have been extensively studied by Michaelis and coworkers (48-50) and by Schubert (63). At pH 7 to 8, Michaelis (48) concluded that the major cobaltous compound formed was cobaltotricysteine. This compound is oxidized in two steps, the first leading to cobaltitricysteine, the second to cobalto-cysteine-cystine which is stable. Ferrous salts are presumed to give the same reactions with the difference that the ferrocystine complex reacts with two molecules of free cysteine so as to furnish free cystine and ferrotricycysteine. In this manner the cysteine is cyclically oxidized to cystine.

The nickel compounds of cysteine are similar to the cobaltous complexes but are resistant to oxidation. Certain of the cobalt-cysteine complexes have been crystallized by Schubert and their chemical properties and interrelationships studied. The structural formulations and the scheme of oxidation proposed by him are given in Fig. 16.

A very stable chelated coordination complex is formed between histidine and cobaltous ion which possesses the unique property of combining reversibly with molecular oxygen (7a, 25a). The histi-

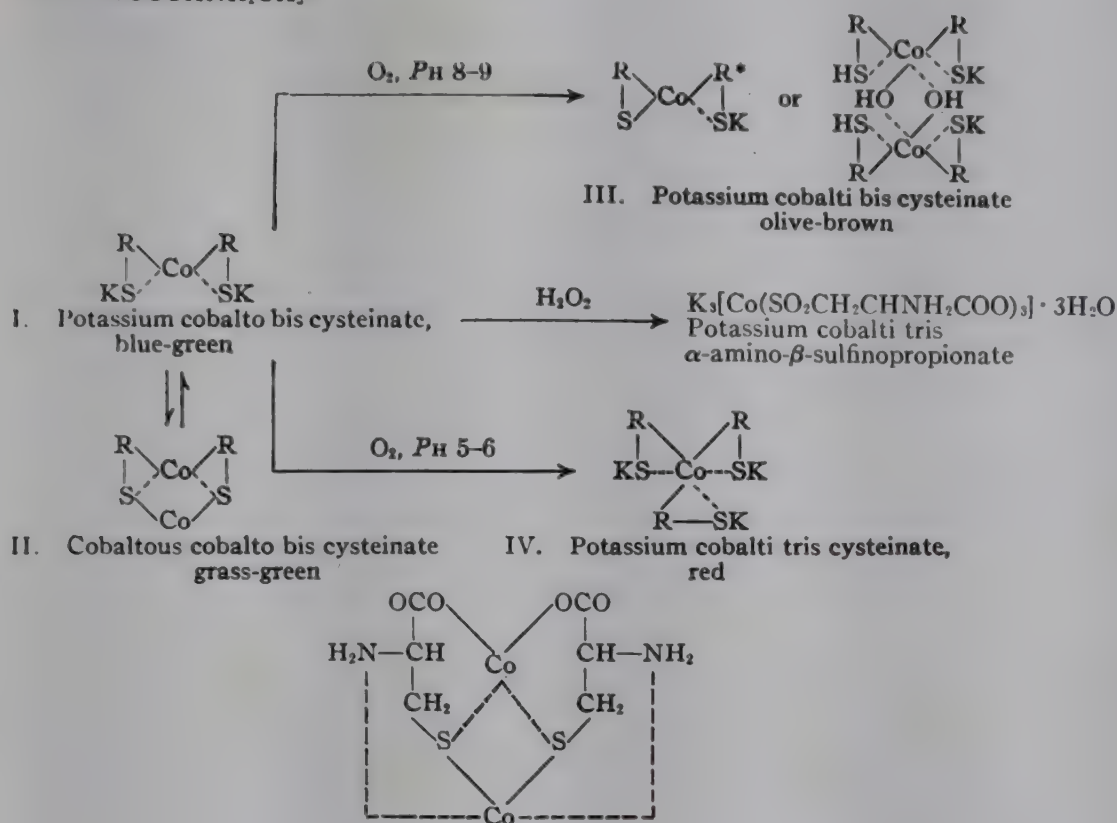
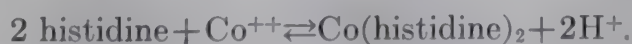


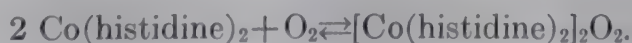
FIG. 16. Cobalt complexes of cysteine. Schubert, M. P.:
J. Am. Chem. Soc., 73:3851 (1931).

* As this compound is hydrated, it may be that the normal six coordination places of the trivalent cobalt are completed by the addition of two molecules of water.

dine complexes of the other transition elements, Ca^{++} , Cu^+ , Ni^{++} , Fe^{++} and Mn^{++} , on the contrary, are not able to combine reversibly with oxygen gas. Other amino acids show much less tendency than histidine to combine with Co^{++} and their reversible complexes with O_2 are quite unstable. Two molecules of histidine combine instantly with Co^{++} according to the reaction:

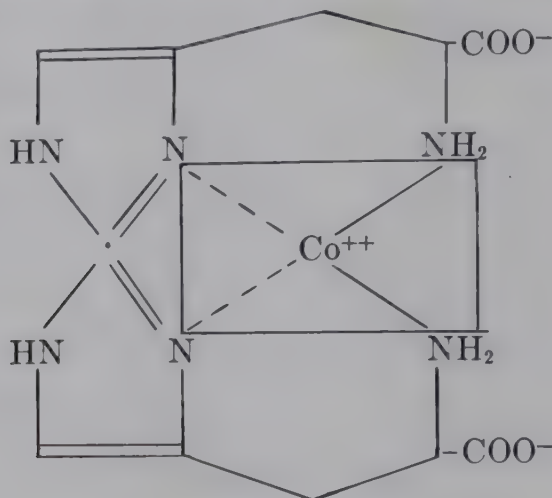


The dissociation constant and heat of the reaction are $K_{25} = 2 \times 10^{-7}$ and $\Delta H = 19 \text{ Kcal. per mole}$. Two molecules of the cobaltodihistidine unite reversibly with O_2 to form a uniquely stable reversible complex. The equation for the reaction is:



For this reaction $K_{25} = 3.6 \times 10^6 \text{ l}^2/\text{mole}^2$ and $\Delta H = -38.2 \text{ Kcal. per mole}$.

Cobalt dihistidine is colored pink, the oxygenated compound is yellow-brown. The structure of the cobaltodihistidine may be represented by the following chelated structural formula (25a):



Cobaltodihistidine

It appears surprising that cobaltodihistidine does not react with carbon monoxide because alkaline Co^{++} does, and carbon monoxide decomposes Co^{++} -cysteine complexes. It is interesting to note that hemocyanin also does not react with carbon monoxide.

Certain substituted histidine compounds (carnosine and anserine) behaved qualitatively like histidine, but 5-methylimidazole did not coordinate with Co^{++} (7a).

The incompletely filled d orbital electrons of cobaltous ion enables it to form stable tetra- and hexa-coordinate complexes. The most stable of these is the covalent octahedral (d^2sp^3) complex (25a). The essential feature of the combination of cobaltohistidine with O_2 is the pairing of single electrons from Co^{++} with single electrons from the oxygen molecule. This pairing accounts for the stabilization of the cobaltous state in the oxygenated complex. This is in agreement with the observed magnetic moments and the stabilization of the bivalent state of the metal. It is noteworthy that the bonds in cobaltodihistidine are essentially ionic, while those in the oxybis(cobaltohistidine) are strictly covalent.

The evidence for this is that the magnetic moment of cobaltohistidine was found to be 4.44 Bohr magneton. The calculated spin moments for three unpaired electrons is 3.88, which is in good agreement with that usually found in ionic cobaltous complexes. The moment for the oxybis(cobaltodihistidine) was found to be zero, which leads to the conclusion that there are no unpaired spins in the oxygenated complex. A similar transition in bond nature

from ionic to covalent is observed in the oxygenation of hemoglobin (54a).

The stability of the histidine complexes is, to a large extent, explained by the resonance of the imidazole ring, which is not possible for other amino acids. A striking feature of cobaltodihistidine is that the Co^{++} is highly resistant to being oxidized to Co^{+++} by O_2 .

The usual amino acids such as alanine, glycine and cysteine are capable of forming complexes composed of three amino acids and one cobalt, thereby filling all available positions. Because of steric hindrance, only two molecules of histidine unite with the cobaltous ion, thus coordinate positions are held open or are filled by weakly bonded groups that are easily replaced by O_2 . This is analogous to the structure of hemoglobin where apparently an imidazole of histidine, in the globin molecule, is held in a position sterically unfavorable to coordination with Fe^{++} and hence is easily replaced by O_2 .

Analogous chelated compounds of Co^{++} capable of combining reversibly with oxygen have been prepared from derivatives of salicyl aldehyde or derivatives of *o*-hydroxyacetophenone and an amine (e.g., ethylenediimine) by Calvin and coworkers (7b).

The copper derivatives, particularly those of thioglycolic acid, but also of cystine and homocystine have been studied by McKittrick (46) under varying conditions of concentration and pH.

4. Metallo-Protein Complexes

Considerable study has been devoted to metallo-protein complexes. The experimental methods employed to demonstrate complex formation consist of electrical migration, colorimetric methods, activity determination with metal-metal ion electrodes (copper, silver, mercury), and by means of the Donnan membrane distribution. The literature on the subject and representative data are given by Schmidt (62). The reader is referred to this work for details.

Great interest in the metallic complexes of proteins stems from the observation that a large number of enzymes require the presence of certain metallic ions to carry on their catalytic function. A widely accepted explanation of this phenomenon is that the associated metal-protein complex represents the active form of the enzyme. The metal serves as an activating prosthetic group. In most instances the combination between metal and enzyme protein appears to be readily dissociable.¹²

¹² Smith and Hanson (J. Biol. Chem., 179, 803 (1949)) report that Mg^{++} is essential for pancreatic carboxypeptidase activity and is so firmly bound to the enzyme that it cannot be removed by dialysis or the procedures involved in its isolation.

TABLE XII
SOME ENZYME SYSTEMS ACTIVATED BY METALLIC IONS

Enzyme	Activating Metal*
<i>Involved in Carbohydrate Metabolism</i>	
Phosphoglucomutase	Mg, Mn, Co
Hexokinase	Mg
Triose mutase (Phosphoglyceromutase)	Mg
Enolase	Mg, Mn, Zn
Phosphopyruvate phosphatase	Mg
Hexodiphosphatase	Mg
Adenylpyrophosphatase	Ca, Mn, Mg, Ba
Isocitric acid dehydrogenase	Mn, Mg
Carboxylase	Mg, Mn
Pyruvic dehydrogenase	Mg, Mn, Co
<i>Phosphatases</i>	
Bone phosphatase	Mg, Mn
"Alkaline" kidney phosphatase	Mg, Mn, Co
Intestinal epithelium phosphatase	Mg
Yeast phosphatase	Mg, Mn, Co, Fe, Ni
"Acid" prostatic phosphatase	Mg, Mn
<i>Miscellaneous</i>	
Liver arginase	Mn, Co, Ni
Intestinal amino peptidase	Mg, Mn
Phenol oxidases	Cu
Carbonic anhydrase	Zn

* Activating metals all present as divalent ions.

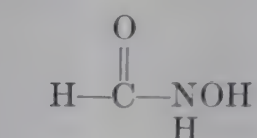
A list of such enzymes and their metal ion activators are given in Table XII.

These examples illustrate the general importance of the interaction with metallic ions for the field of protein chemistry. The group or groups in the enzyme protein which link with the cation is not definitely known in any single case. Much remains to be done to obtain a clear understanding of the manner in which the metal ions control the biological and catalytic functions of proteins.

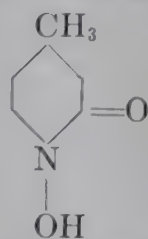
Unique and specific iron-binding proteins have been discovered in blood serum and in egg white. These are a β_1 -pseudoglobulin component of blood serum, named siderophilin, and conalbumin of egg white (17a, 61a). Siderophilin has been isolated from human and swine blood plasma (28a, 35a). These proteins combine specifically with ferrous ion in the presence of oxygen and bicarbonate to yield salmon-pink complexes during which the iron is converted to the ferric state. In the absence of oxygen and in the presence of excess bicarbonate, the addition of ferrous ion results in the formation of a yellow complex. Addition of neutralized ascorbic acid converts it to the pink complex (17a, 61a). The full color of the iron siderophilin, once developed, is not diminished by equilibration with a CO_2 -free gas phase. It is stable above pH 6.0 and breaks up at low pH's, being half decomposed at pH 4.8 and completely decomposed at

pH 4.0. In the siderophilin complex, protein, iron and HCO_3 occur in the molecular proportions of 1:2:2.

Evidence for the nature of these specific iron-protein complexes has been obtained from the observation that similar compounds are formed by N-hydroxypyridine derivatives, hydroxylamine, and hydroxamic acids. Formulas of some examples are given below:

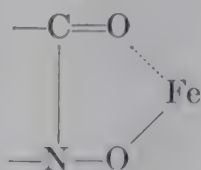


hydroxamic acid



N-hydroxy-4-methyl
pyridine-2

Aspergillic acid, N-hydroxy pyridine derivatives (N-hydroxy-4-methyl pyridine-2, N-hydroxy-5-bromopyridine-2), containing the cyclic hydroxamic acid grouping, and hydroxylamine bind ferric ions to yield deep salmon-colored complexes with the same absorption spectrum maximum as the above proteins (460–465 $\text{m}\mu$). The presence of bicarbonate is required to form the colored iron complex with hydroxylamine, but not with aspergillic acid nor the N-hydroxypyridine compounds. This suggests that in the complexes formed with siderophilin, conalbumin and hydroxylamine, the bicarbonate serves to form a hydroxamic acid type of grouping, equivalent to the carbonyl group adjacent to the hydroxylated nitrogen already present in the cyclic hydroxamic acid grouping. The oxygen when ferrous ion is added, may be presumed to oxidize it to ferric ion. The complex formed may be represented by the chelated structure below:



In further agreement with this conception the two proteins, like hydroxamic acids generally, yield colored complexes with copper. However, the proteins do not show the same degree of specificity for binding copper as they do for iron.

XII. BINDING OF ANIONS BY PROTEINS¹³

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As in the case of metal-protein complexes, data on the binding of anions by proteins are primarily qualitative in character and have been obtained primarily in connection with studies of the transport function of blood proteins (2a, 13a). Recently, however, quantitative investigations on the binding of various organic anions have also been initiated and the results obtained have been treated from the point of view of the law of mass action (34b, 34c).

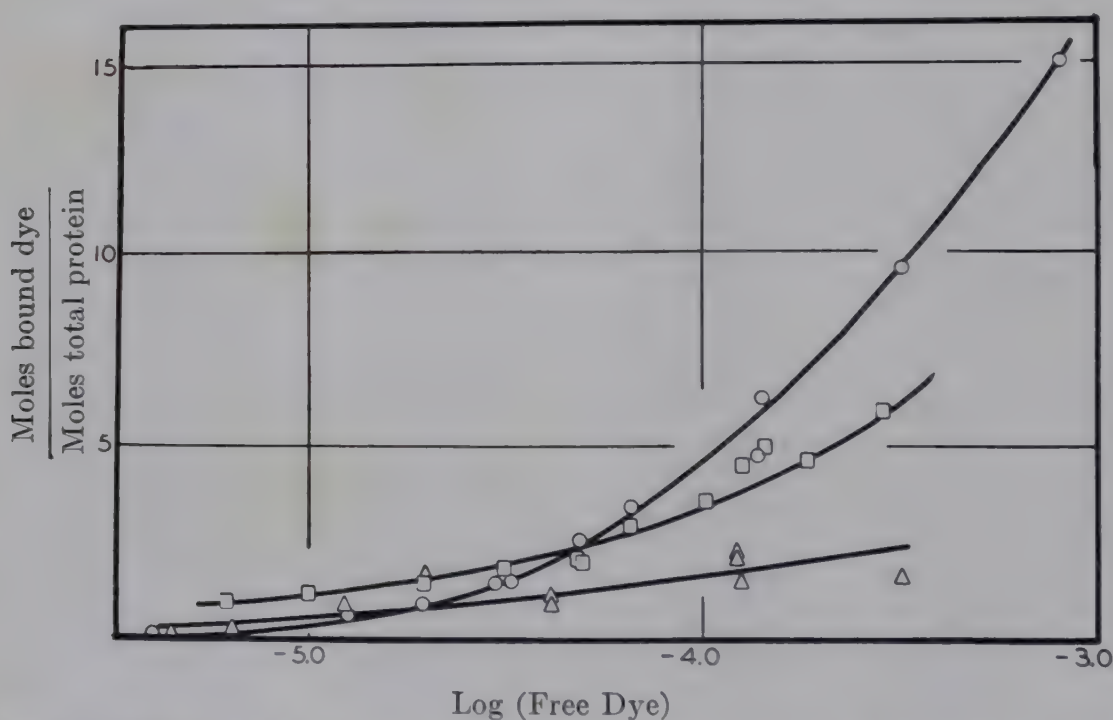


FIG. 17. Binding of sulfonate compounds by bovine serum albumin at pH 5.7: ○, methyl orange; □, azosulfathiazole; △, amaranth.

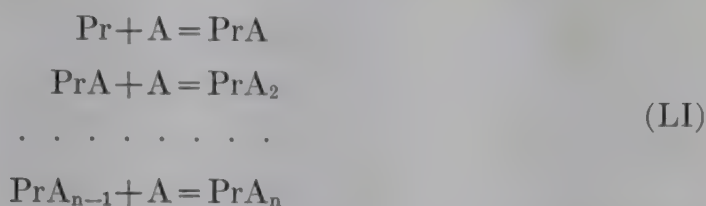
1. Application of the Law of Mass Action

The results on the binding of three sulfonated organic ions, singly, doubly, and triply charged, respectively, by bovine serum albumin are summarized in Fig. 17. For an analysis in terms of the law of mass action it is convenient to plot r , the moles of bound ion per mole of protein, as a function of $\log A$, the logarithm of the concentration of the free anion. It is immediately evident from the figure that many molecules of dye may be bound by a single protein

¹³ For a discussion of the thermodynamics (entropy, enthalpy) of binding, and the relation between protein structure and the ability to bind ions see (34d).

molecule. In fact, for methyl orange, the singly-charged anion, bindings of as high as 15 ions have been observed.

A rigorous treatment of the data by means of the law of mass action must take into account the formation of such multiple complexes. The formulation of the necessary equations is quite analogous to that described for the rigorous treatment of metal-protein complexes. If Pr, represents the protein and A, the anion, the following equilibria must be present:



As in the case of metal-protein complexes, one may write an equilibrium constant for each step in equation LI,

$$\frac{[\text{PrA}_n]}{[\text{PrA}_{n-1}][\text{A}]} = K_n \tag{LII}$$

and a product of constants such as the following

$$\frac{[\text{PrA}_n]}{[\text{Pr}][\text{A}]^n} = K_1 K_2 \dots K_n = \prod_{i=1}^{i=n} K_i. \tag{LIII}$$

For r , an equation analogous to XLV for metal complexes would be obtained and may be written in generalized notation as follows:

$$r = \frac{\sum_{n=1}^{n=m} n \left(\prod_{i=1}^{i=n} K_i \right) [\text{A}]^n}{1 + \sum_{n=1}^{n=m} \left(\prod_{i=1}^{i=n} K_i \right) [\text{A}]^n} \tag{LIV}$$

The simplest situation one may encounter in protein binding is that in which anion is bound to the same kind of group on the protein and in which a bound ion exerts no electrostatic influence on succeeding bindings. In such a situation the strength of attachment would be the same for each bound anion. Hence the relative values of the successive equilibrium constants of equation LII would be determined solely by statistical factors and would be given by the relation

$$K_n = \frac{m - (n - 1)}{n} \frac{1}{K_{\text{PrA}}} \tag{LV}$$

where K_{PrA} is a specific binding constant which depends on the nature of the anion as well as on the character of the protein. Where the statistical effect is predominant and the equilibrium constants are given by equation LV, the relatively involved equation LIV may be reduced to the very simple linear form

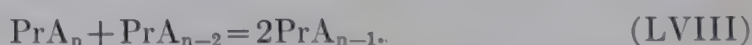
$$\frac{1}{r} = \frac{1}{m} \left[\frac{K_{PrA}}{[A]} + 1 \right]. \quad (LVI)$$

This statistical relation is a very good approximation to the quantitative data on the binding of singly-charged anionic dyes such as methyl orange, orange I and orange II as well as for certain metal ions such as calcium. However, for dyes with two or three charges, the statistical distribution of constants is unable to account for the observed binding data. Obviously it is necessary to include the electrostatic interactions previously neglected. This has been done (34b, c) by considering the free energy of the binding process, ΔF° , to be composed of three parts,

$$\Delta F^\circ = -RT \ln K = \Delta F_{stat.} + \Delta F_{specific} + \Delta F_{elect.} \quad (LVII)$$

where $\Delta F_{stat.}$ represents the statistical contribution, $\Delta F_{specific}$ the effect of the intrinsic binding constant, K_{PrA} , and $\Delta F_{elect.}$, the effect of electrostatic interactions.

To focus attention on the effect of the charge of the anion alone it is convenient to consider the transfer of one dye ion from one complex to another of lower degree, as is represented in the equation



For this reaction it has been shown (34b) that the electrostatic free-energy change is given by the expression obtained from the Debye-Hückel theory

$$\Delta F_{elect.} = -\frac{Nz^2e^2}{D} \left(\frac{1}{b} - \frac{K}{1+K \cdot a} \right) \quad (LIX)$$

where N is Avogadro's number; z , the number of charges on A ; e , the electronic charge; D , the dielectric constant of the medium; b , the radius of the protein molecule; a , the "distance of closest approach" of a charged ion to the protein; and K is given by

$$K = \left(\frac{4\pi Ne^2}{1000DkT} \right)^{1/2} \Gamma^{1/2} \quad (LX)$$

where k is the Boltzmann constant, T , the absolute temperature, and Γ twice the ionic strength of the medium.

From the equation LIX it is clear that the electrostatic effect, depending on the square of z , becomes increasingly important with increasing charge on the anion. Since the anion and protein have the same charge, the electrostatic interaction is a repulsive one,

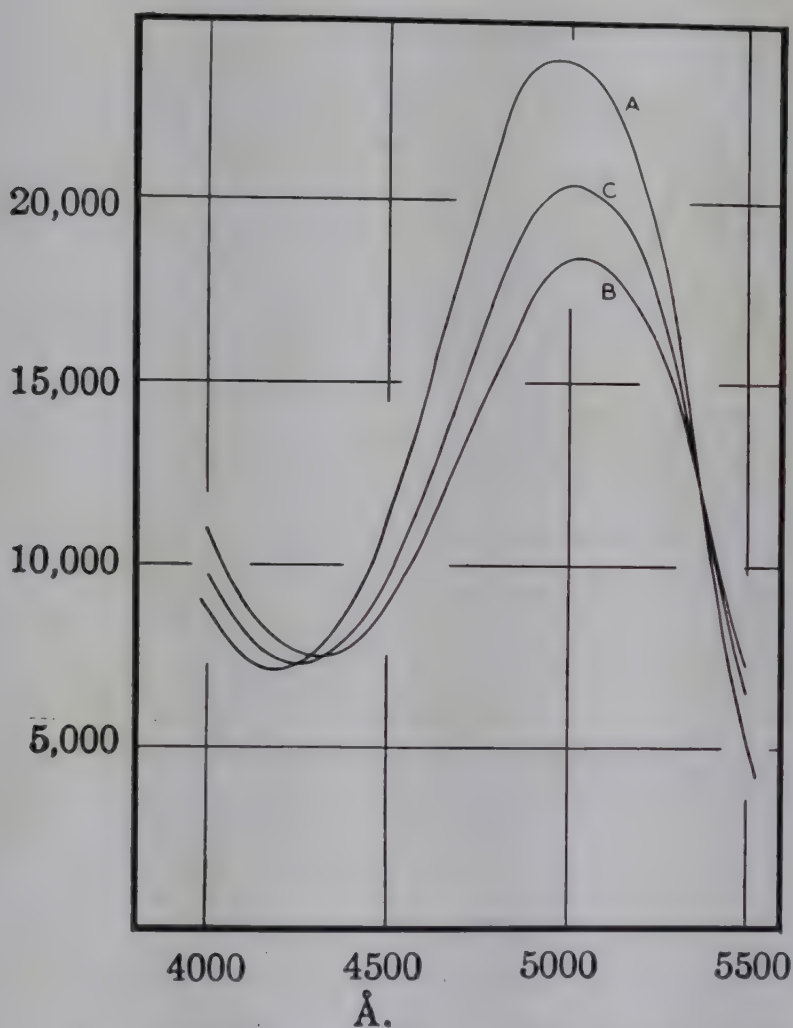


FIG. 18. Absorption spectra of azosulfathiazole: A, in buffer at pH 6.92; B, in buffer containing bovine albumin (0.2%), pH 6.90; C, in buffer containing bovine albumin and *p*-aminobenzoic acid ($1.34 \times 10^{-2} M$), pH 6.45.

and the protein tends to bind fewer of the more highly charged anions as the anion concentration increases. Thus the curvature of the graphs in Fig. 17 should be less for azosulfathiazole (two charges) than for methyl orange (one charge), and that for amaranth (three charges) should be smallest. The observed behavior is not only in qualitative agreement with the theoretical explanation but also quite close quantitatively. By making reasonable choices for the radii of the protein and of the dye anions it is possible to cal-

culate the electrostatic free-energy change from equation LIX. This information in addition to an empirical evaluation of a single constant, K_{PrA} , the intrinsic binding constant, enables one to evaluate the individual constants, K_n , and ultimately, r , the moles of bound anion per mole of total protein. Quite satisfactory agreement has been found between the theoretical curves so calculated and the observed experimental values (Fig. 18).

2. Changes in Spectra

Since bound anions are in a different environment from that in the aqueous solution it is not too surprising to find that the spectra of bound dyes change in protein solution (Fig. 18). The changes may be used to calculate the extent of binding (34a). In solutions of low protein concentration which contain both bound and unbound dye, the absorption of light may be expressed by the relation

$$\log (I_0/I) = \epsilon_1 c_1 d + \epsilon_2 c_2 d \quad (\text{LXI})$$

where ϵ_1 is the molecular extinction coefficient of the unbound anion, c_1 , its concentration, ϵ_2 and c_2 corresponding values for the bound anion, d the depth of the optical path and $\log (I_0/I)$ the optical density. If we define $\epsilon_{\text{apparent}}$ by the relation

$$\epsilon_{\text{apparent}} = \frac{\log (I_0/I)}{(c_1 + c_2)d} \quad (\text{LXII})$$

then it can be shown readily that α_i , the fraction of the anion which is free, may be calculated from the expression.

$$\alpha_i = \frac{\epsilon_{\text{apparent}} - \epsilon_2}{\epsilon_1 - \epsilon_2} \quad (\text{LXIII})$$

Spectral changes may also be used as an indicator of the displacing ability of various uncolored anions toward the bound colored anion (34a). The optical method has been used also to investigate the effect of pH on binding by proteins (34c).

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Chapter VIII

CRITERIA OF THE PURITY OF A PROTEIN

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I. INTRODUCTION

METHODS generally employed in defining the purity of a simple chemical compound, cannot be applied to proteins. The difficulties

encountered with proteins are due to their unstable nature and macromolecularity. For instance, proteins are decomposed at their melting temperature and thus the best criterion for purity of simple organic materials, melting point determination, cannot be used for substances of protein nature.

The elementary composition of most proteins shows that they have very similar percentages of carbon, hydrogen, oxygen and nitrogen. Analysis of these elements, hence, gives no information as to the differentiation of one protein from another.

It is now well recognized that crystallinity is an inadequate criterion of purity. Evidence by other criteria has accumulated indicating that many crystalline proteins are actually mixtures. A crystalline protein may be contaminated by amorphous material or crystals of other material. The formation of a mixed crystal or a solid solution is not uncommon in proteins. Herriott, *et al.* (26) showed that crystalline pepsin preparations, which are mixed crystals, can be resolved to yield a single crystalline component.

In order to define how pure a protein is, we therefore depend upon certain special properties of the molecule. The best measures of the purity of a protein are based upon the physico-chemical or biological properties of its molecules. If one finds that a protein possesses uniformity of these properties, it enhances the probability that the protein is a pure substance. Individual criteria of purity are not sufficient, and even the agreement of a large number of criteria only increases the probability but does not insure purity; a single new criterion may perhaps show that the protein sample is in fact a mixture. The physico-chemical properties which provide the more satisfactory evidences of purity are ultracentrifugation, diffusion, electrophoresis, and solubility; the biological properties, enzymic, hormonal or virus activity (63, 68). Among these measurements, solubility is considered to be the best single criterion for the purity of proteins since it is based on the theory of the phase rule, like the melting point universally employed for a purity test in organic chemistry.

II. BIOLOGICAL ACTIVITY

If the protein under investigation possesses biological activity, this property may be employed to ascertain its purity if a sensitive and specific bioassay procedure exists. The protein should have constant biological activity if it is pure. Close attention must be paid, however, to the technique of bioassay. Although the contaminating proteins may have no biological potency, they may influ-

ence the activity of the physiologically active material. For example, protamine increases the blood-sugar reducing activity of insulin if the hormone is contaminated with it. For this reason, it is necessary to investigate whether contaminants that are likely to be present exert any non-specific effect on the results of bioassay.

In isolating biologically active material from animal fluid or tissue, the impurities in the final product are often found to possess certain physiological activities that may tend to enhance or depress the biological potency of the substance sought. Therefore, in such a case, constant biological activity cannot be accepted as a criterion of purity. If there are sensitive methods of assay to detect the presence of impurities, they should be applied in ascertaining whether or not this particular contaminant is present. For example, in the process of isolating the growth hormone (50) from ox anterior pituitary, two biologically active contaminating proteins (thyrotropic and adrenocorticotropic hormones) may be present: one synergizes and the other antagonizes the growth-promoting action. In order to ascertain the biological purity of the growth hormone, constant growth-promoting potency and the absence of thyrotropic and adrenocorticotropic activities must be established.

It is known that the immunological reaction (see Chap. XI) is very sensitive in detecting minute quantities of an antigen. When the nature of a possible impurity is suspected, anaphylatic or precipitin reactions may be applied in ascertaining the purity of the product. Chester (7) has demonstrated the presence of normal proteins in tobacco mosaic virus preparations using an anaphylactic test. Chow (8) was able to conclude from the precipitin reaction that his follicle-stimulating hormone preparation was free from the luteinizing component.

III. DIFFUSION

If a column of solution is layered above a solvent in a vertical tube of uniform cross-section so that a concentration gradient exists between the solution and the solvent, the process of free diffusion tends to decrease the gradient to zero. The rate of diffusion is expressed by Fick's equation:

$$\frac{ds}{dt} = -DA \frac{dc}{dx} \quad (I)$$

where ds/dt is the quantity of material which diffuses in a unit time, A the cross-sectional area of the column, and dc/dx the

concentration gradient. D is the diffusion constant and is a function of the size and shape of the molecule. Equation I applies to a particular level in the column (x) for which the values of s , A and c are as given at time t .

There are two principal methods currently employed to determine the diffusion constant (see Chap. VI). The first is the classical free diffusion technique (33, 34) which measures the quantity dc/dx as a function of the distance, x , by means of a scale photograph or modified schlieren method. The second method involves the diffusion of the solute through a sintered glass disc (58), the pores of which must be large enough to allow the molecules to pass freely and yet sufficiently small to prevent the occurrence of convection.

The sintered glass disc method is useful in estimating the monodispersity of biologically active proteins. If the amounts of protein diffused in equal time intervals are constant and if the diffused protein fractions contain the same biological potency, it is inferred that the protein is homogeneous. Of course, the conclusion depends upon the specificity and accuracy of the bioassay.

In the free diffusion method, the monodisperse nature of a protein is generally estimated by comparison of the experimental diffusion curve with the ideal diffusion distribution curve. If the two curves are identical, the material may be considered to be monodisperse; deviations on the other hand can be used to determine the degree of polydispersity of the protein. Since the rate of diffusion depends upon the size and shape of the molecule, it is possible that the diffusion constant of a nearly spherical but large molecule could be the same as that of a protein which is elongated and which has a smaller molecular weight. Furthermore, a pure protein solution contaminated with very low molecular weight materials may give misleading results by free diffusion experiments. The diffusion curve of certain proteins are observed to be asymmetrically deviated from the normal curve. Neurath (61) has described such a type of curve for tobacco mosaic virus protein. Bevilacqua, *et al.* (3) attributed the skewed curve to the effect of the extremely elongated shape of the materials; it cannot be explained simply by the presence of several components. Sometimes, a preparation is known to be heterogeneous and yet the diffusion curve does not deviate from the normal curve. For instance, Bridgman (5) found that several glycogen samples known to be polydisperse behaved as homogeneous materials by the test of free diffusion. Caution must be employed in the interpretation of

the diffusion curves as a test for the monodisperse nature of a protein.

IV. ULTRACENTRIFUGATION

Large molecules like proteins can easily be sedimented in a high centrifugal field. The specific rate of sedimentation, s , in relation to the molecular weight, M , may be expressed by the equation:

$$M = \frac{RTs}{D(1 - V\rho)} \quad (\text{II})$$

where R is the gas law constant, T the absolute temperature, D the diffusion constant, ρ the density of the solution, and V the partial specific volume of the protein. The specific sedimentation rate, s , is defined by the equation

$$\frac{dx/dt}{\omega x^2}$$

where ω is the angular velocity and x the distance from the center of rotation. In general, the movement of the material during sedimentation is observed by optical methods similar to that used in electrophoresis. If the preparation is homogeneous with respect to molecular size a single boundary results; if more than one boundary occurs during ultracentrifugation it is certain that the protein is polydisperse. A single boundary, however, does not necessarily indicate that the protein is pure (see also Chap. VI).

It has been suggested by Svedberg (75) that molecular weights of proteins tend to fall into groups which are a multiple of 17,600. Regardless of how valid this hypothesis may be, it nevertheless suggests that many proteins may have similar molecular weights. Since the accuracy of the sedimentation velocity method of determining molecular weights is approximately 5%, as shown by Stanley and Lauffer (79), it becomes difficult to determine each component in the ultracentrifuge if a mixture contains several proteins of nearly the same molecular weight.

There are other difficulties in employing this method as a test of purity of proteins. In the case of very elongated molecules (69) a single boundary may be obtained in the ultracentrifugation of several proteins although they are actually different in length. Lundgren (52) has shown that the denatured form of thyroglobulin sediments at a rate similar to that of the native protein in the absence of electrolytes, but appears polydisperse in the presence of salt. The observations of Gralén (23) and Kabat (28) that the

sedimentation rate changes with protein concentration, led to the discovery of the phenomenon of spurious sharpness in the boundary. This is caused by the slowest moving particles that tend to travel in the solvent region. The occurrence of such phenomena makes it difficult to judge the homogeneity of the preparation. In conclusion: "To deduce identity in the chemical nature of a protein from identity in its sedimentation velocity is a danger clearly envisaged by all those familiar with the methods" (10).

V. THE MOVING BOUNDARY METHOD OF ELECTROPHORESIS

When an electric current passes through a medium containing charged colloidal particles, the particles bearing a negative charge migrate toward the positive electrode and *vice versa*. This phenomenon is called electrophoresis. Although the first electrophoretic experiment was performed by Reuss over one hundred forty years ago, experiments with proteins using the moving boundary technique were first carried out by Hardy (25) in 1905. Hardy allowed an opalescent protein solution to flow gently under a fluid of the same electrolyte content in a U tube, forming a sharp boundary between the colloid and the solvent. Since then many modifications of the U tube have been introduced¹ but it was not until 1937, after the improvements made by Tiselius (85), that the moving boundary method of electrophoresis² became a powerful and useful tool in the investigation of proteins. The advances made by Tiselius may be briefly summarized as follows: (a) the suggestion of operating electrophoresis experiment at 0° to minimize the convection currents caused by the density gradient inside the U tube; (b) the introduction of a sensitive optical system to observe the movement of the boundary; and, (c) the employment of flat rectangular cells with different compartments to increase the resolving power and separation efficiency.

1. Methods of Observing Boundaries

Two types of optical methods are generally used for the measurement of concentration gradients, namely, the light-absorption method and the refractive index methods. The former method, based on the fact that proteins have an absorption maximum at about 270 m μ in the region of ultraviolet radiation, has been applied successfully by Svedberg and Tiselius (74, 84) in electrophoresis

¹ See historical summary in (80).

² For general treatment of this subject, see (1, 2, 78, 92, 45).

experiments. Due to the difficulties of obtaining quartz parts of high optical quality for the construction of the U tube, the light-adsorption method has not been widely used.

The scale method and the schlieren method are those based upon the refractive properties of the colloids. The scale method was originally suggested by Lamm (34) for diffusion studies and later applied to electrophoresis (29, 87). Although the principle of the scale method is simple and its operation comparatively economical,

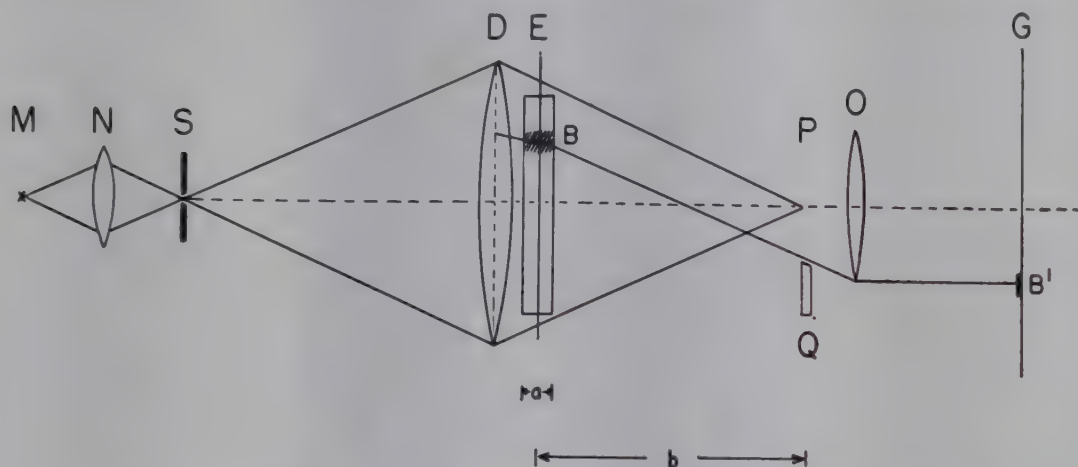


FIG. 1. Arrangement for observation of the boundaries with the schlieren or shadow method.

the analysis of the scale line photographs is laborious and time-consuming. This method is therefore not used in routine electrophoresis experiments.

a. The schlieren method

The optical arrangement introduced by Tiselius (85) is based upon the experiments of Foucault and Toepler³ who discovered that small differences of refractive index in a given medium can be detected on a screen as schlieren or shadows. Figure 1 shows a diagram of the optical arrangement for schlieren or shadow method. An image of a horizontal slit is formed in the plane, *p*, by the schlieren lens, *D*. The slit, *S*, is illuminated by the lamp, *M*, and condenser, *N*. In the plane, *p*, a schlieren diaphragm, *Q*, with a sharp, horizontal edge may be moved vertically with a micrometer. The electrophoresis cell, *E*, is placed as near as possible to the lens, *D*. The camera objective, *O*, placed immediately behind the schlieren diaphragm is focussed on the cell and the image is formed on a ground-glass or photographic plate at *G*.

³ For a detailed discussion of the Foucault-Toepler schlieren method, see (66).

Where there is no boundary in the cell, the image of the cell at G appears uniformly illuminated. If, however, a boundary, B, is present in the cell, the refractive index decreases with the height through the boundary. The pencil of light through this region will be deflected downward. On raising the diaphragm, the deflected pencils are intercepted and thus they appear as a dark band, B', on a light background. A typical example of the use of the simple

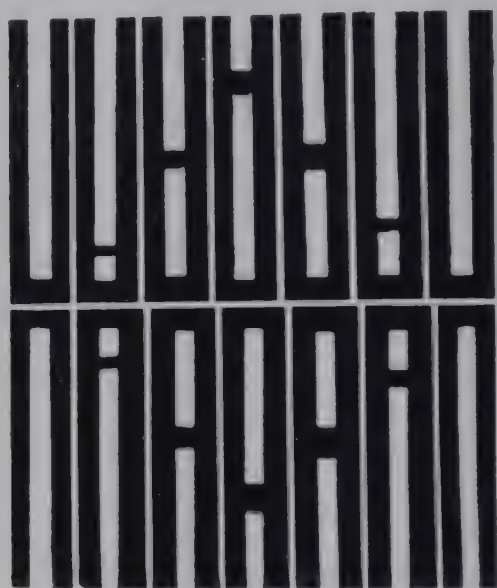


FIG. 2. Schlieren bands of pregnant mare serum gonadotropin. Exposures were made at 20-minute intervals and the current was reversed after the fourth exposure. Li, C. H., Evans, H. M., and Wonder, D. H.: *J. Gen. Physiol.*, 23:733 (1940).

schlieren method for following the moving boundaries in an electrophoretic experiment with the pregnant mare serum gonadotropin is shown in Fig. 2. The horizontal dark bands are the shadows of the boundaries between the protein in a phosphate buffer and the pure buffer. The exposures were made at 20-minute intervals; the upper and lower photographs are the ascending and descending boundaries in the U-tube, respectively.

It is clear from the preceding discussion that maximally deflected light pencils correspond to the maximum concentration gradient, and this is proportional to the refractive gradient, dn/dh ; n is the refractive index and h the height of the concentration gradient. When the schlieren diaphragm, Q (Fig. 1), continuously moves slowly upward, the dark band, B', in G becomes broader and broader until the whole field becomes completely dark. If different photographs are taken at different fixed positions of the schlieren diaphragm, a series of dark bands of increasing breadth are formed.

Figure 3 represents an example of such photographs for a single protein. It is, therefore, apparent that the gradient, dn/dh , of refractive index varies from zero to a maximum, and back to zero.

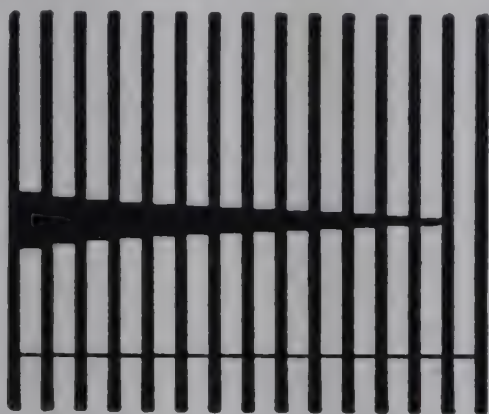


FIG. 3. Schlieren bands, for different schlieren diaphragm settings, of a single protein. Longworth, L. G.: *Indust. & Engin. Chem., Anal. Ed.*, 18:219 (1946).

Hence, the displacement, Δ , of the schlieren diaphragm is proportional to dn/dh or

$$\Delta = ab \frac{dn}{dh} \quad (\text{III})$$

where a is the dimension of the channel parallel to the optical axis and b is the optical distance from the center of the channel to the schlieren diaphragm.

b. The Longworth scanning method

Longworth (36) has developed a scanning method operated mechanically to obtain a photograph relating the refractive index

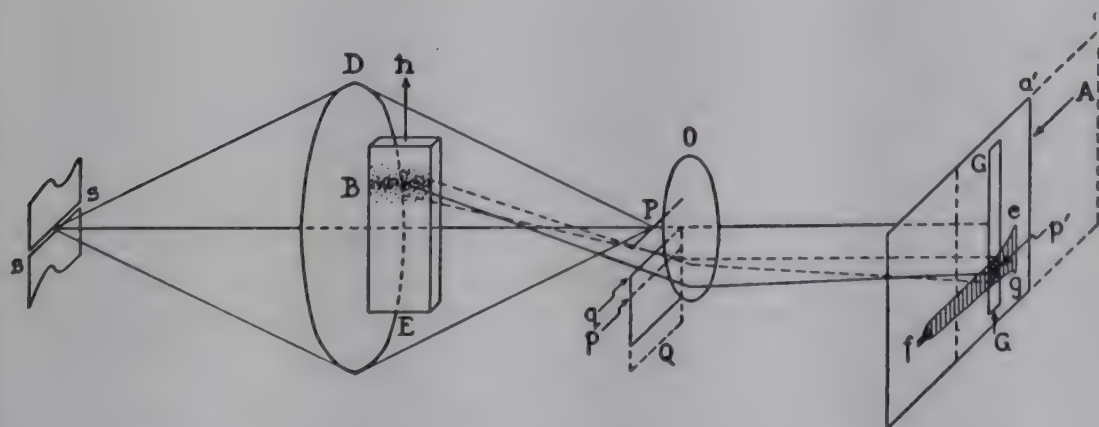


FIG. 4. Diagram of the schlieren scanning method for the photographic recording of gradients of refractive index. MacInnes, D. A., and Longworth, L. G.: *Colloid Chemistry*, 5:387 (1944).

gradient with the height in the column of the solution. The method is illustrated in Fig. 4. As in the case of the simple schlieren method, an image of the illuminated slit *S* is formed by the lens, *D*, at the plane, *P*. The image of the electrophoresis cell at *G*, formed by the camera lens, *O*, is masked by a vertical slit of 0.2 mm width, and a photographic plate is moved in the direction of the arrow at a constant rate across this slit. The diaphragm, *Q*, is moved upward by the same mechanism at a rate proportional to the velocity of the moving photographic plate. The variation of the gradient of the boundary, *B*, in the cell, *E*, is therefore represented by the density of the shaded area *e-f-g*. Since the photographic plate, *A*, was in position *a* at the time the schlieren diaphragm was at *p*, a section of the band *p'* appears at *f* when the plate has been moved to *a'*. The simple schlieren bands are narrow sections through the area *e-f-g*.

Both the position and the magnitude of the refractive index gradient existing in the boundary can be determined from the contour area *e-f-g*. From equation III, it can be demonstrated that the area *A*, under the curve is

$$A = \Delta dh = abrm(dn) = abrm(n_1 - n_0) \quad (IV)$$

in which $(n_1 - n_0)$ is the refractive index increment of the protein solution, *r* the ratio of the speed of the plate movement to that of the diaphragm, and *m* the magnification of the camera. Since the refractive index increment is proportional to the protein concentration, *C*,

$$C = \frac{kA}{abrm} \quad (V)$$

where *k* is the specific refractive increment of the particular protein under consideration. Thus, it is apparent that the electrophoretic patterns obtained by the Longworth scanning technique may be used to determine the protein concentration in the boundary if the protein is the only substance contributing to the gradient.

In addition to the scanning device of Longworth, Philpot (64) and Svensson (76, 78) have developed the cylindrical lens method (See Chap. VI). The Philpot-Svensson technique is convenient for visual observation during an experiment, since the pattern may be viewed directly on the ground-glass of the camera. It is generally agreed that the greatest disadvantage of this method is the difficulty in obtaining adequately corrected cylindrical lenses.

2. The Electrophoresis Apparatus

Since Tiselius (85) introduced the new technique in electrophoretic studies, his original apparatus has been greatly improved by Longworth (37, 55, 45) in this country and Svensson (78) in Sweden. The following description of the apparatus is essentially that given by Longworth.

The complete electrophoresis apparatus is shown in Fig. 5. The optical bench⁴ consists of two steel channels, C, bolted together

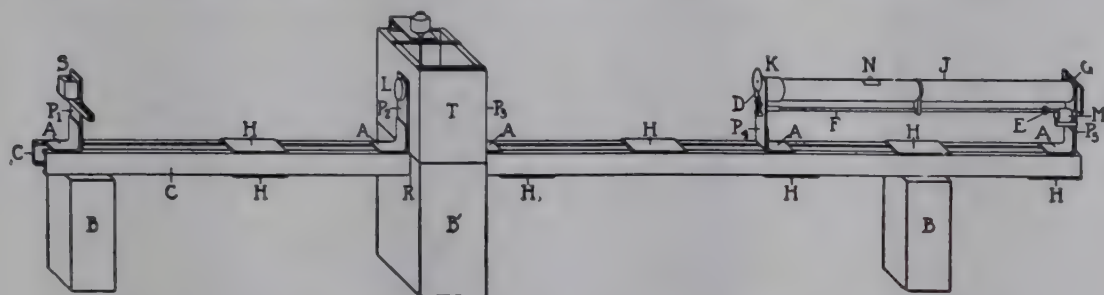


FIG. 5. Scale diagram of complete apparatus. Longworth, L. G.: *Indust. & Engin. Chem., Anal. Ed.* 18:219 (1946).

by means of the plates, H. The bench is supported on two concrete blocks, B, and passes through a trough, R, in the block, B, that carries the thermostat, T. Each component of the optical system is carried by a steel plate, P, which is bolted through slotted holes to the vertical faces of the steel angles, A. S is the horizontal slit, L the schlieren lens, D the sharp-edged diaphragm, G the ground glass or photographic plate, and M the slow-speed shaft of the synchronous motor and reducing unit for controlling the movements of the photographic plate and the schlieren diaphragm. The electrophoresis cell is placed in the thermostat as close as possible to the lens, L. A compensation device, not shown in Fig. 5, is mounted on the side wall of the thermostat.

a. Electrophoresis cells

The electrophoresis cell and electrodes are held together in a metal support as shown in Fig. 6. The cell consists of a U tube⁵ of rectangular cross-section which is divided into four sections; these may be slid over one another by a rack-and-pinion system. The

⁴ The design requires a rigid optical bench 20 feet in length. Recently Swingle (83a) described an electrophoresis apparatus using parabolic mirrors with the advantages of reducing the optical bench to 16 feet or less.

⁵ The volume of this U-tube is about 10 ml. Tiselius (89) has also introduced a large cell (200–300 ml.) for separation purposes and a micro-cell with a capacity of only 2 ml.

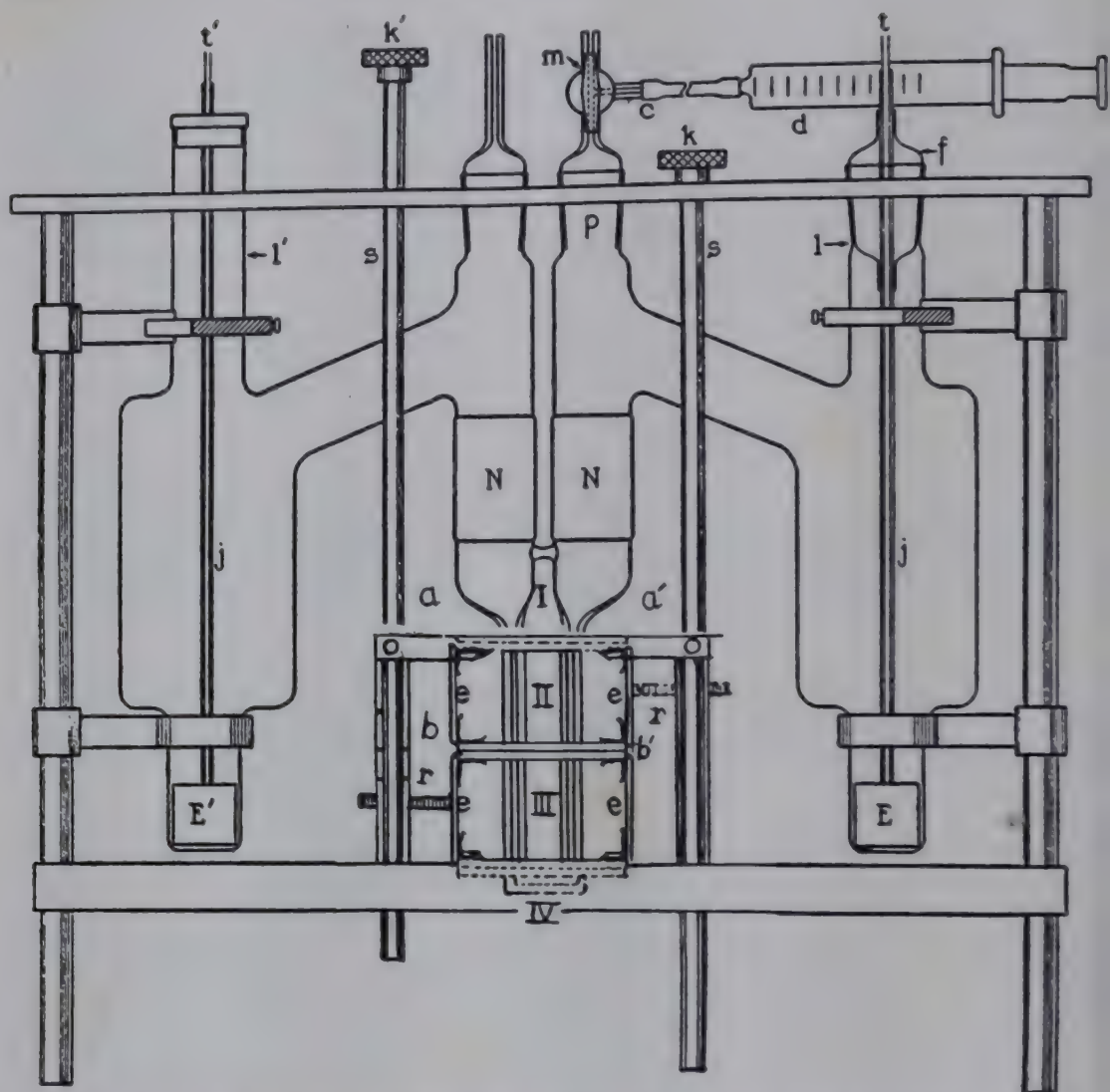


FIG. 6. Electrophoresis cell, electrode vessels, and support.

racks, *r*, are attached to the shafts, *s*; by turning the knurled knob, *k*, the rack presses against a metal insert, *e*, which communicates the pressure to the edges of the horizontal glass plates. The center sections of the cell are interchangeable with a tall section. This double length section, introduced by Longsworth, *et al.* (39), is illustrated in Fig. 7. The cell is connected to the side arms of the electrode vessels by rubber or neoprene sleeves, *N*. The electrodes, *E* and *E'*, are made by winding a flat and a corrugated strip of sheet silver together into a tight spiral, the ends of spirals being anchored to a hollow silver core with silver screws (37). Such electrodes are capable of carrying currents of at least 30 milliamperes for long periods of time without evolution of gas.

b. Thermostat

One of the important improvements introduced by Tiselius is to keep the electrophoresis cell at the temperature of maximum den

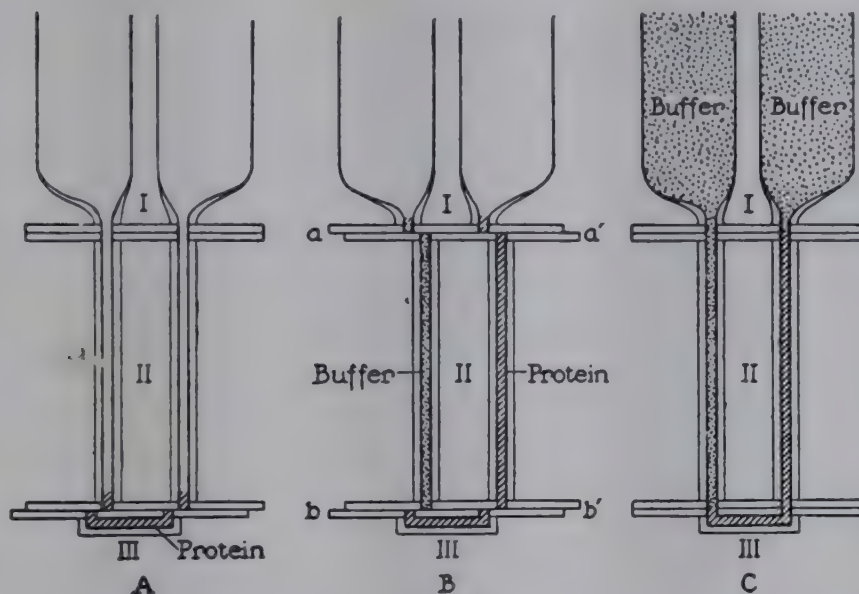


FIG. 7. Diagrams illustrating the initial formation of the boundaries in the Tiselius electrophoresis cells with the tall center section. Longworth, L. G.: *Chem. Revs.*, 30:323 (1942).

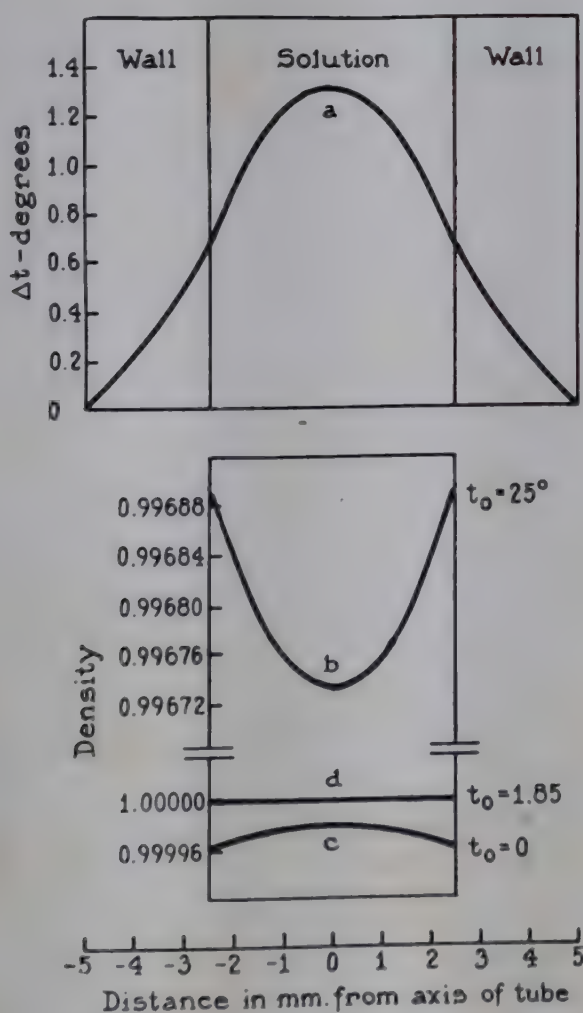


FIG. 8. Distribution of temperature and density in a salt solution, in a cylindrical tube, during passage of electric current. Longworth, L. G., and MacInnes, D. A.: *Chem. Revs.*, 24:271 (1939).

sity of water so that the risk of heat convection at the boundary becomes minimum. Longsworth and MacInnes (37) have computed the effect of temperature on the density of a 0.1 M sodium acetate solution in a cylindrical tube of 5 mm. internal and 10 mm. external diameter after passing through it a current of 0.006 ampere, according to the equations of Mooney (53). It is apparent from Fig. 8 that if the thermostat temperature is regulated at 1.85° , the density gradient would be a minimum, as indicated by the horizontal line in Fig. 8. Therefore, the thermostat should always be maintained at or near this temperature.

c. Compensation device

The purpose of a compensation device is twofold: to bring the initial boundary into view from the horizontal glass plates before the onset of electric current, and to move the entire solution of the cell at a constant speed in order to assign to any boundary a desired apparent velocity. The original device of Tieslius (85) consists of a clockwork motor in connection with an ebonite plunger in one of the electrode vessels. The fact that it is not possible to alter the compensation rate or direction during an experiment without terminating the experiment limits its application. Longsworth and MacInnes (37) introduced one closed electrode vessel and forced the buffer solution into the closed side from a syringe to displace the solution in the cell at a desired rate. The syringe is operated by a threaded rod and a synchronous clock motor.

3. Method of Operation

a. Preparation of protein solutions

As already mentioned, the boundary is formed in the electrophoresis U-tube between the protein solution and a supernatant liquid of the same composition with respect to all other constituents but free of protein. The protein solutions, therefore, must be dialyzed against the same buffer for an adequate time so that the conductivities of the buffer solution outside and the protein solution inside the membrane do not differ by more than 2% or even less. In general, 12–15 ml. of protein solution is dialyzed against 2 liters of the buffer in a cold room ($2-5^{\circ}$) for at least 24 hours.⁶ Experience has shown that equilibrium is readily attained within 12 hours if the outside buffer solution is stirred constantly during the dialysis. At the end of dialysis, the protein solution may need

⁶ Reiner and Fenichel (64a) have described a mechanical dialyzer and claimed that it is possible to equilibrate protein solutions against a buffer within two hours.

to be filtered or centrifuged if it appears to contain any precipitate. The buffer solution is used subsequently to fill the electrode vessels and other connections.

The optimum protein concentration is determined by the object of the experiment. If the primary purpose is to determine the electrophoretic *mobility* of a single protein, its concentration should be low but sufficient to produce a sharp boundary. For ordinary proteins (*e.g.*, egg albumin) a 0.2% solution is recommended. On the other hand, if the main object is to establish the electrophoretic *homogeneity* of a preparation of unknown purity or to estimate the concentration of various components in a mixture, higher concentrations (1 or 2%) are preferable.

b. The choice of buffer

The choice of buffer depends upon the desired pH and also upon the protein to be investigated. For instance. Longsworth (42) found that human plasma contains an α_1 component which can be separated from the albumin in sodium diethylbarbiturate buffer at pH 8.6, while the α_1 -globulin appears only as a shoulder on the albumin peak in phosphate buffer at pH 7.7. In the case of horse plasma, phosphate buffer gave a more satisfactory pattern than in

TABLE I
COMPOSITION, pH AND CONDUCTIVITY OF SOME BUFFER SOLUTIONS

Buffer	Ionic Strength	pH	Conductivity		Reference
			Temperature Degrees	$\lambda \times 10^3$	
0.2 N glycine-0.1 N HCl	0.1	2.5	1.49	5.76	(1)
0.02 N NaAc-0.2 N HAc 0.08 N NaCl	0.1	3.6	0.62	5.37	(1)
0.02 N NaAc-0.1 N HAc-0.08 N NaCl	0.1	4.0	1.5	3.45	(2)
0.1 N NaAc-0.15 N HAc	0.1	4.5	1.2	3.92	(1)
0.1 N NaAc-0.5 N HAc	0.1	5.6	0.58	7.84	(1)
0.02 N NaC-0.02 N HC-0.08 N NaCl	0.1	6.0	0.59	5.40	(1)
0.025 M NaH ₂ PO ₄ -0.025 M Na ₂ HPO ₄	0.1	6.8	0.5	2.77	(3)
0.004 M NaH ₂ PO ₄ -0.032 M Na ₂ HPO ₄	0.1	7.7	0.0	2.62	(4)
0.008 M NaH ₂ PO ₄ 0.064 M Na ₂ HPO ₄	0.2	7.7	0.0	4.81	(4)
0.04 N NaV-0.02 N HV 0.06 N NaCl	0.1	8.2	0.0	4.58	(4)
0.1 N NaV-0.02 N HV	0.1	8.6	0.0	3.03	(4)
0.1 N NaOH-0.6 N glycine	0.1	9.0	0.0	3.48	(4)
0.1 N NaOH-0.125 N glycine	0.1	10.8	0.58	3.50	(1)

NaAc—Sodium acetate.

HAc—Acetic acid.

NaC—Sodium cacodylate.

HC—Cacodylic acid.

NaV—Sodium diethylbarbiturate.

HV—Diethylbarbituric acid.

(1) Ward, W. H., unpublished.

(2) Li, C. H., unpublished.

(3) Tiselius, A., and Horsfall, F. L., Jr.: *J. Exptl. Med.*, 69:83 (1939).

(4) Longsworth, L. G.: *Chem. Revs.*, 50:323 (1942).

the diethylbarbiturate buffer. Moore (56) has subsequently confirmed the conclusion of Longsworth that the proper solvent for plasma analysis varies with the species and should be determined experimentally. The ionic strength of the buffer may vary between 0.05 to 0.20; the desired ionic strength depends chiefly upon the protein concentration. In general, the buffer concentration should increase with increasing protein concentration, thereby diminishing boundary disturbances resulting from the inequalities of buffer distribution due to the Donnan equilibrium. With a 0.2% protein solution an ionic strength of 0.05 may be used; with a 1% solution, the ionic strength of the buffer should be at least 0.1 and often 0.2. Table I presents a list of buffers⁷ generally used for the electrophoretic experiments.

The pH of the buffer and that of the protein solution may be determined with a glass electrode. The conductance measurements are carried out in the electrophoresis thermostat at the temperatures used in the actual experiments. Longsworth and coworkers (38) introduced a new type of conductivity cell for protein solutions, which has the advantage of reducing foam formation. A simple Wheatstone bridge is adequate in precision to measure the conductance of the protein and buffer solutions.

c. Procedure

All ground surfaces of the cells, which previously have been dried completely, are well greased with a suitable mixture of vaseline and paraffin oil. The sections of the cells are placed one above the other, each section being moved a little under pressure until the air between the greased sections is completely pressed out. The combined sections of the cell are then placed on the frame of the metal support (Fig. 6). Sections III and IV are then filled with the protein solution to a level slightly above the plane *b-b'*; section III is pushed to one side and the excess protein solution in section II is removed and rinsed with the buffer solution 2 or 3 times. The remainder of the cell⁸ and the attached electrode vessels are then filled with the buffer. The top of the cell (section I) is next connected to the side arms of the electrode vessels with rubber sleeves. It is important to ascertain that there are no air bubbles in

⁷ For other buffers, see (54), p. 278.

⁸ In the case of a double-length cell (Fig. 7), the bottom section III is filled with the protein solution slightly above the plane *b-b'* and then pushed to the left as shown at A in Fig. 7. One side of section II is filled with protein solution and the other side rinsed and filled with the buffer, both sides being filled above the plane *a-a'*. Section II is then moved to the right, after which section III returns to the center as shown in B of Fig. 7. The excess protein solution is then rinsed out and both sides of this section are filled with the buffer.

the cell and the electrode vessels. If bubbles are present, they may be removed by a long fine needle; the needle should not touch the anterior or posterior walls of the cell, since scratching will destroy their optical homogeneity.

The electrodes E and E' are next inserted into the electrode vessels. Saturated potassium or sodium chloride (20 ml.) is then introduced around each electrode through the silver tubes *t* and *t'*. More buffer solution is added to the electrode vessels to the levels *l-l'*. A small piece of narrow rubber tubing is inserted to close the tube *t* in the closed electrode to insure air-tightness; care should be taken to exclude air bubbles as the ground glass stopper, *f*, is seated.

The apparatus is next placed in the thermostat. The syringe, *d*, with attached rubber tube, *c*, filled with buffer, is connected to the threeway stopcock, *m*, as shown in Fig. 6. The syringe is clamped in position in the compensator. The electrical leads are connected to the tubes *t* and *t'*. It is essential to observe that the white electrode is always used as the positive pole. Therefore it is advisable to reverse the polarity of the electrodes with each successive experiment. After the apparatus has been in the thermostat for at least 30 or 45 minutes to attain thermal equilibrium, the stopcock is turned through 90° from the position shown in Fig. 6 and the U-tube is opened slowly by turning the knurled knobs *k* or *k'*. The boundaries between the protein and buffer solutions are then formed and are brought out from behind the opaque horizontal plates of the cell by starting the compensator. After the boundaries are visible, the cell is isolated by turning the stopcock *m* through 90°.

The electric current is next turned on, the voltage being chosen so that not over 5 watts are dissipated by the cell, *e.g.* 250 volts and 20 milliamperes. Since the heating effect varies with wattage, potentials exceeding this limit may cause convection within the cell. In general, experiments with a buffer of ionic strength 0.1 employ a potential gradient of 4-6 volts per cm. After some time, the boundary in one side of the center section of the cell will migrate upward and that in the other side downward. At suitable intervals, several scanning exposures⁹ may be taken; the timing depends upon the migration rate of the protein. The current is also read several times during the experiment.

⁹ Eastman Kodak orthochromatic plates are satisfactory for use. If solutions are completely opaque in the visible range, infra-red light with infra-red sensitive plates have been used successfully (91).

When the experiment is finished, the current is broken and the U-tube is closed by shifting the center cell(s) to the side. The apparatus is lifted out of the thermostat; the electrodes are removed and kept under water. The electrode vessels are emptied and washed with distilled water. The contents in each section of the U-tube can then be aspirated by a long stainless steel needle attached to a syringe. If the contents are to be discarded, the cell need not be closed at the end of the experiment; the U-tube is emptied simply by turning it upside down. The cell is finally dismantled and the grease between sections is carefully removed with tissue paper or cotton. The final cleaning is performed with 2% duponol¹⁰ solution.

4. Boundary Anomalies

a. The δ and ϵ boundaries

In the classical investigation of Tiselius (85) a false resting boundary was observed on the rising side of the U-tube. This boundary, later recognized by Tiselius and Kabat (88) as the δ boundary, is caused by a salt gradient superimposed upon a protein gradient. Longsworth and MacInnes (40) confirmed these observations and showed further that a corresponding false boundary, named the ϵ boundary, is also present on the descending side. A typical example of these boundaries is shown in Fig. 9; the electrophoretic pattern was obtained with a lactogenic hormone solution in a sodium acetate buffer.

The explanation for the presence of these false boundaries has been given by Longsworth and MacInnes (40). Figure 10 illustrates the relative positions of the boundaries in the electrophoresis cell. When passage of a current causes the descending boundary to move from a to d , the intervening volume, V_d , has a buffer composition, B' . This composition has been "adjusted" by the passage of electric current, usually to a value different from that of the original buffer, B . Thus, the ϵ boundary is formed between two solutions of the same buffer, each with a different concentration. In the rising side, there is a similar but more complicated adjustment of the composition of the protein solution which replaces the buffer as the boundary moves from a' to r . The resulting δ boundary, between the solutions P and P' , is much more visible than the ϵ boundary, since the former involves a gradient of protein concentration in addition to a salt concentration gradient, whereas the latter does not. It is

¹⁰ Duponol is a detergent manufactured by E. I. Du Pont de Nemours and Company, Inc.

evident that these false boundaries may not appear in protein solutions of sufficiently low concentration. It has been shown (40) that appropriate selection of the concentration of the buffer solution may avoid the formation of these boundaries.

b. Non-identity of the rising and descending boundaries

When an electric current of potential gradient F passes through a vertical tube of one square cm. cross sectional area containing an electrolyte solution of concentration, C , the change of concentration is given by the Kohlrausch equation

$$\frac{\partial C}{\partial t} = - \frac{\partial(uFC)}{\partial x} \quad (\text{VI})$$

where u is the mobility, t the time and x the distance. For an ideal case, u and F are independent of x ; otherwise equation VI becomes

$$\frac{\partial C}{\partial t} = -uF \frac{\partial C}{\partial x} - uC \frac{\partial F}{\partial x} - FC \frac{\partial u}{\partial x} . \quad (\text{VII})$$

Suppose that only u varies with x and that u is a linear function of x . Then $u = Ax + B$, in which A and B are constants. We obtain

$$\frac{\partial C}{\partial t} = FAC - (Ax + B)F \frac{\partial C}{\partial x} . \quad (\text{VIII})$$

The solution of this equation (78) may be written:

$$C = e^{-FA t} \Phi[e^{-FA t}(Ax + B)] \quad (\text{IX})$$

where Φ , the arbitrary function, is determined by the concentration distribution at the beginning ($t = 0$):

$$C_0 = \Phi(Ax + B). \quad (\text{X})$$

It is apparent that if F and A have the same sign, the boundary would gradually spread out with increasing t , whereas if F and A have opposite signs the boundary would become sharpened. That is to say, migration into a region of increasing mobility causes a blurring of the boundary, while migration into a region of decreasing mobility causes a sharpening of the boundary.

The predictions deduced from equations IX and X have been observed in actual experiments. It may be noted (Fig. 10) that the electric field in P is greater than that in B due to a smaller specific conductance in P than in the adjusted buffer solution. Hence, the boundary d migrates to a region of increasing mobility and becomes

diffuse, as shown in Fig. 9b. For similar reasons, the boundary r tends to remain sharp, as illustrated by Fig. 9a.

c. Non-identity of the mobilities of the rising and descending boundaries

It was found that the conductance of solution P' is less than that of solution P (Fig. 10). Accordingly, the potential gradient below the δ -boundary turns out to be smaller than that above it. Boundary

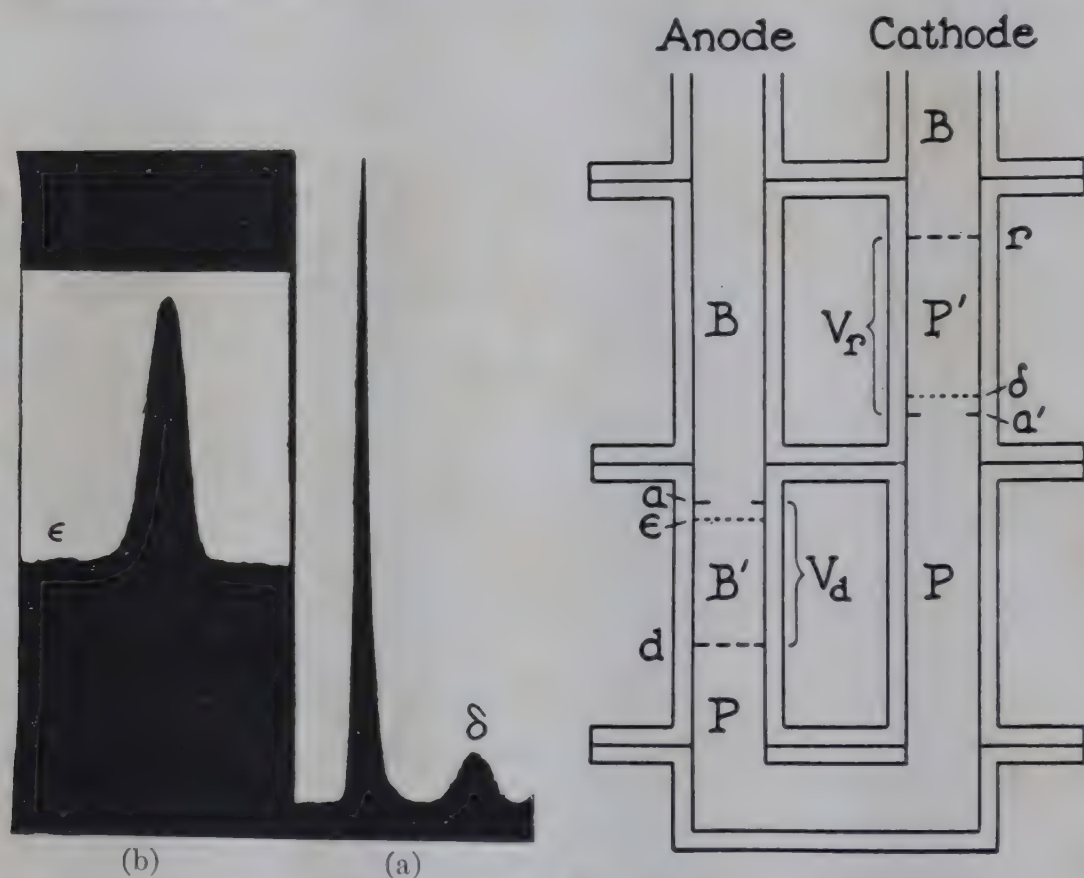


FIG. 9. Electrophoresis patterns of a lactogenic hormone solution in pH 4.0 acetate buffer illustrating the presence of δ and ϵ boundaries.

FIG. 10. Diagram of the electrophoresis cell illustrating the relative position of the boundaries. MacInnes, D. A., and Longworth, L. G.: *Colloid Chemistry*, 5: 387 (1944).

r must, therefore, be expected to move more rapidly than boundary d . This anomaly is generally observed. For instance, when the protein concentration is about 1%, the rising boundary is always found to migrate 5 or 10% faster than the descending one.

It may be seen from the preceding discussion of boundary anomalies that the major cause of these anomalies is due to differences of specific conductance. Therefore, a proper choice of the buffer may reduce these disturbing phenomena. Svensson (77) has derived an equation to express the conductivity change across the boundary; namely,

$$\frac{\Delta\lambda}{\lambda} = \frac{\Delta u'}{u'} - \frac{C(1 + \Delta u'/u')^2}{B u} \quad (\text{XI})$$

where λ is the conductivity, u' the mobility of the buffer ion, u the mobility of the colloid, C the colloid concentration and B equals $\epsilon c'/u'$. As a first approximation, the term $\Delta u'/u'$ may be neglected and equation XI becomes

$$\frac{\Delta\lambda}{\lambda} = - \frac{B_{\text{colloid}}}{B_{\text{buffer}}} . \quad (\text{XII})$$

From these equations, it is clear that the requirements for the most suitable buffer are that it have a high salt concentration and its component ions low mobilities. Similar conclusions have been previously reached by Longworth and MacInnes (40).

5. Electrophoretic Mobility

a. General theory

Let us assume a spherical particle in a potential gradient. F , carrying a net charge, q . The resultant electric force is qF . A viscous force is also operating to oppose the migration of this particle. Following Stokes' law, we calculate this retarding force to be $6\pi\eta r v$, where η is the viscosity of the medium, r the radius, and v the velocity of the sphere. At a steady state, these two forces become equal, and

$$v = \frac{qF}{6\pi r \eta} . \quad (\text{XIII})$$

Since the mobility, u , equals v/F ,

$$u = \frac{q}{6\pi r \eta} . \quad (\text{XIV})$$

The potential of a sphere with charge q , in an environment of dielectric constant D , is:

$$\zeta = \frac{q}{D r} . \quad (\text{XV})$$

Substituting this equation into equation XIV, we obtain

$$u = \frac{\zeta D}{6\pi \eta} . \quad (\text{XVI})$$

It must be remembered that the use of equation XVI is restricted to the case of an isolated particle. In the presence of other charged

particles, *i.e.*, salts, the ζ -potential is no longer simply q/Dr ; ζ reduces to smaller values as the salt concentration increases. The effect is due to the formation of an ion atmosphere (double layer) caused by the accumulation of charged particles of opposite sign. If the double layer had a thickness of d and the outer sphere had the charge $-q$, the potential on the outer surface would possess a value of $-q/D(r+d)$. Therefore, the total potential becomes

$$\zeta = \frac{q}{Dr} - \frac{q}{D(r+d)} = \frac{q}{Dr} \cdot \frac{1}{(1+r/d)}. \quad (\text{XVII})$$

According to Debye and Huckel (12), the reciprocal of the thickness of the ion double layer, κ , is a function of the ionic strength, μ , of the medium.

$$\kappa = \frac{1}{d} = \sqrt{\frac{8\pi N e^2}{1000 D k T}} \cdot \sqrt{\mu} \quad (\text{XVIII})$$

where e is electronic charge, N Avogadro's number, k Boltzmann's constant, and the other terms have their usual significance. Thus, equation XVI becomes

$$u = \frac{q}{6\pi r \eta} \cdot \frac{1}{(1+\kappa r)}. \quad (\text{XIX})$$

This expression is valid provided the radius of the sphere is smaller than the thickness of the double layer; that is to say, that it is not independent of the size and shape of the particles. Henry (24) has introduced a function, $f(\kappa r)$, satisfying the conditions for a sphere of any size

$$u = \frac{q}{6\pi r \eta} \cdot \frac{f(\kappa r)}{(1+\kappa r)}. \quad (\text{XX})$$

Henry's function may be computed from the following expression derived by Gorin (18a):

$$f(\kappa r) = 1 + \frac{(\kappa r)^2}{16} - \frac{5}{48} (\kappa r)^3 - \frac{(\kappa r)^4}{96} + \frac{(\kappa r)^5}{96} - e^{\kappa r} \left[\frac{12}{96} (\kappa r)^4 - \frac{(\kappa r)^6}{96} \right] \int_{\infty}^{\kappa r} \frac{e^{-t}}{t} dt. \quad (\text{XXa})$$

Thus, when $\kappa r \gg 1$, $f(\kappa r)$ becomes 1.5 and when $\kappa r \ll 1$, $f(\kappa r) = 1$.

b. Measurements

The total quantity, p , of protein transported in the electric field of potential gradient, F , for t , seconds in a U-tube of cross-sectional area, A , is given by:

$$p = uACFt \quad (\text{XXI})$$

where C is the concentration of protein in grams per ml. Since the quantity, p , is determined by following the motion of a boundary between the protein solution and the buffer, it may be put equal to CXA in which X is the observed distance that the boundary has moved. Thus equation XXI becomes

$$u = \frac{CXA}{ACFt} = \frac{X}{Ft} \quad (\text{XXII})$$

It is apparent, therefore, that the electrophoretic mobility of a protein is defined as the distance moved per second in a unit potential gradient by an average particle in the body of the protein solution (40).

The potential gradient, F , is determined by the specific conductance, λ , of the protein solution, the current, i , in amperes, and the cross-sectional area, A , of the channel, hence:

$$F = \frac{i}{A\lambda} \quad (\text{XXIII})$$

The quantity, A , may be estimated by the following procedure: The ground surfaces of the dried cell are greased and one end of the cell is closed with a plane ground glass plate. The greased cell together with the glass plate is first weighed empty, then with one limb filled with mercury, and finally with both limbs filled. The weight differences are converted into volumes. The height of the cell is next measured to within 0.1 mm. The division of the volumes by the height gives the cross-sectional areas. Or, less precisely, the cross-sectional area may be measured with caliper and feeler gauges.

It is generally accepted (40) that the movement of the descending boundary should be used for the calculation of the mobility.¹¹ For ordinary purposes, the migration rate of the boundary may be read directly from the ground glass in the camera. At least four readings should be taken at varying time intervals. The readings

¹¹ The unit of mobility is expressed as cm. per sec. per volt per cm., or sq. cm. per sec. per volt.

are plotted against the time and the mobilities are computed from the slopes of these straight lines. The quantity, X , in equation XXII may also be determined from the electrophoretic pattern directly; *i.e.*, X equals the distance between the maximum ordinates of the descending boundary and that of the initial or ϵ boundary. For the highest precision, the ordinate should be that which bisects the area under the gradient curve. If this procedure is followed, several exposures must be taken and the "maximum" ordinate of the curve determined photometrically (41, 43).

c. The change of mobility with pH and ionic strength

According to equation XX, an alteration of pH tends to change the charge, q , of a protein and, in turn, its mobility. On the other

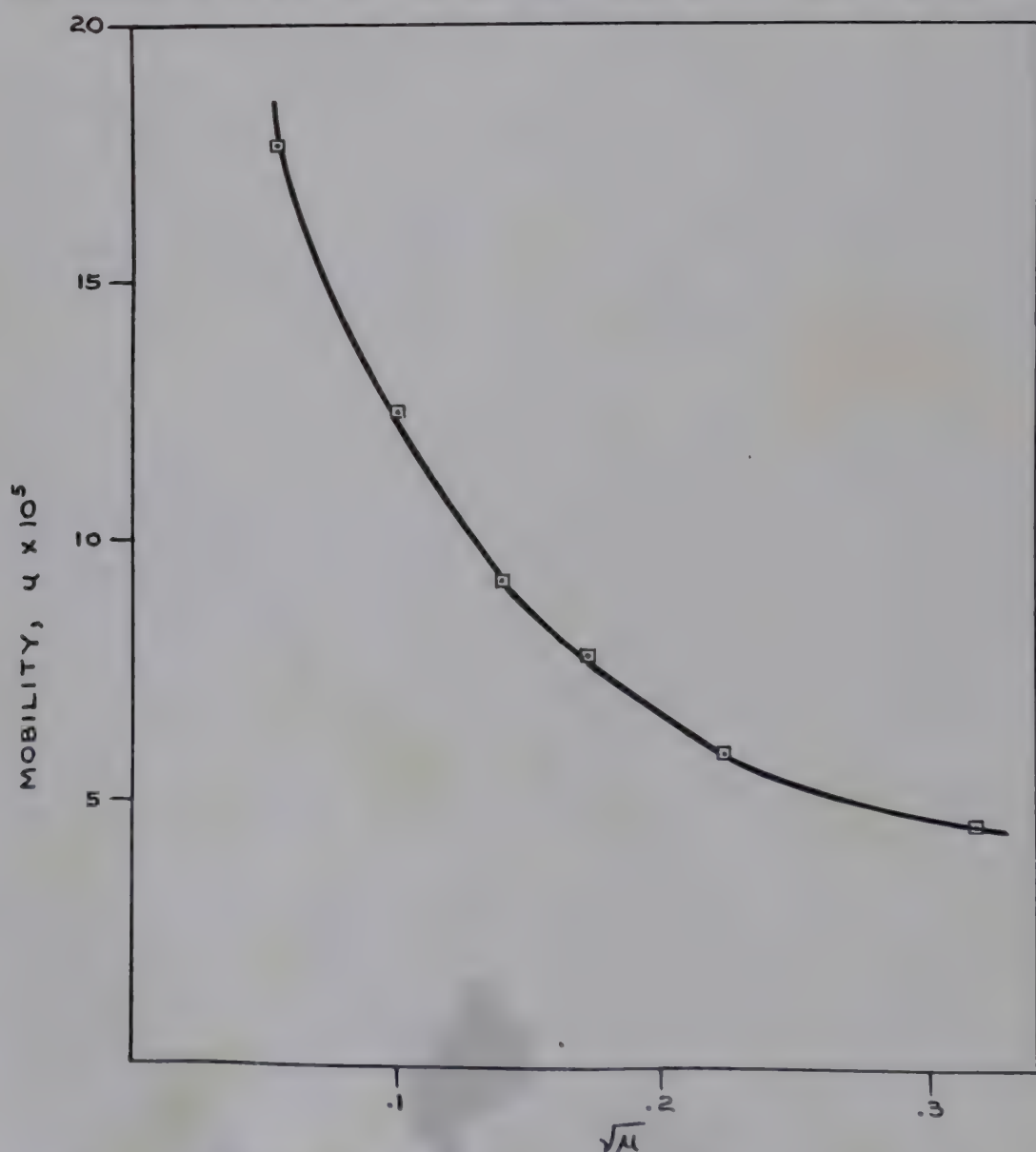


FIG. 11. The mobility of lactogenic hormone in pH 4.0 acetate buffer as the function of ionic strength at 1.5°.

TABLE II

ELECTROPHORETIC MOBILITY AND ISOELECTRIC POINT OF SOME PROTEINS
AS OBTAINED BY MOVING BOUNDARY METHOD OF ELECTROPHORESIS IN PHOSPHATE BUFFER

	Ionic Strength	Temper- ature Degrees	Mobility		Isoelec- tric Point pH	Refer- ence
			pH	u		
				10 ⁵		
Cytochrome C	0.1	0	7.0	+ 2.6	10.65	(1)
Lysozyme	0.1	0	6.5	+ 3.6	10.5	(2)
Ribonuclease (yeast)	0.055	0	6.8	+ 1.4	7.8	(3)
Globin (human)	0.1	0	6.8	+ 0.7	7.5	(4)
Growth Hormone	0.10	1.5	7.0	- 0.2	6.85	(5)
γ-Globulin (human)	0.10	0	6.9	- 0.1	6.4	(6)
Phosphorylase b (rabbit muscle)	0.10	2	7.0	- 2.5	5.8	(7)
Lactogenic Hormone	0.055	1.5	7.0	- 5.75	5.73	(8)
Fibrinogen (human)	0.1	0	6.9	- 1.5	5.40	(6)
Carbonic anhydrase	0.1	0	7.0	- 2.0	5.30	(9)
Insulin	0.1	0.2	7.56	- 5.45	5.30	(10)
β-Globulin (human)	0.1	0	6.9	- 3.0	5.20	(6)
β-Lactoglobulin	0.02	20	7.0	-14.0	5.19	(11)
Papilloma virus* (rabbit)	0.1	0	7.0	- 1.2	5.0	(12)
Crotoxin	0.1	1.5	7.0	- 4.97	4.71	(13)
Albumin (human)	0.1	0	6.9	- 5.5	4.64	(6)
Ovalbumin†	0.1	0	6.79	- 5.16	4.58	(14)
Thyroglobulin (hog)	0.02	20	7.02	-16.1	4.58	(15)
Bushy Stunt virus‡	0.02	0	5.47	- 4.59	4.11	(16)

* In veronal buffer.

† In cacodylate buffer.

‡ In acetate buffer.

(1) Theorell, H.: *J. Am. Chem. Soc.*, **63**:1820 (1941).(2) Alderton, G., Ward, W. H., and Fevold, H. L.: *J. Biol. Chem.*, **157**:43 (1945).(3) Rothen, A.: *J. Gen. Physiol.*, **24**:203 (1940).(4) Munro, M. P., and Munro, F. L.: *J. Biol. Chem.*, **150**:427 (1943).(5) Li, C. H., Evans, H. M., and Simpson, M. E.: *J. Biol. Chem.*, **159**:353 (1945).(6) Stenhagen, E.: *Biochem. J.*, **32**:714 (1938).(7) Green, A. A.: *J. Biol. Chem.*, **158**:315 (1945).(8) Li, C. H., Lyons, W. R., and Evans, H. M.: *J. Am. Chem. Soc.*, **62**:2925 (1940).(9) Petermann, M. L., and Hakala, N. V.: *J. Biol. Chem.*, **145**:701 (1942).(10) Hall, J. L.: *J. Biol. Chem.*, **139**:175 (1941).(11) Pedersen, K. O.: *Biochem. J.*, **30**:961 (1936).(12) Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W.: *J. Biol. Chem.*, **142**:193 (1942).(13) Li, C. H., and Fraenkel-Conrat, H.: *J. Am. Chem. Soc.*, **64**:1586 (1942).(14) Longworth, L. G.: *Ann. N. Y. Acad. Sci.*, **41**:267 (1941).(15) Heidelberger, M., and Pedersen, K. O.: *J. Gen. Physiol.*, **19**:95 (1935).(16) MacFarlane, A. S., and Kekwick, R. A.: *Biochem. J.*, **32**:1607 (1938).

hand an increase or decrease of electrolyte concentration affects the thickness of the ion atmosphere, thus causing the mobility to change with the ionic strength of the medium. It may be seen in Fig. 11 that the mobility of lactogenic hormone decreases as the sodium chloride content in 0.02 *M* acetate buffer (pH 4.0) increases. The mobilities of egg albumin (90) and hemoglobin (11) were also found to be retarded by an increase of ionic strength. This retarding effect seems to follow the decrease of the thickness of the ion double layer as given by equation XVIII.

Since a protein is of amphoteric nature, its net charge may be-

come positive or negative depending upon the pH of the solvent. At a certain pH, the net positive charge equals the net negative charge; this pH is called the isoelectric point (25) of the protein. When a protein dissolves in a buffer at its isoelectric point, the boundary between the protein solution and the buffer will not move in an electric field. A plot of electrophoretic mobility as a function

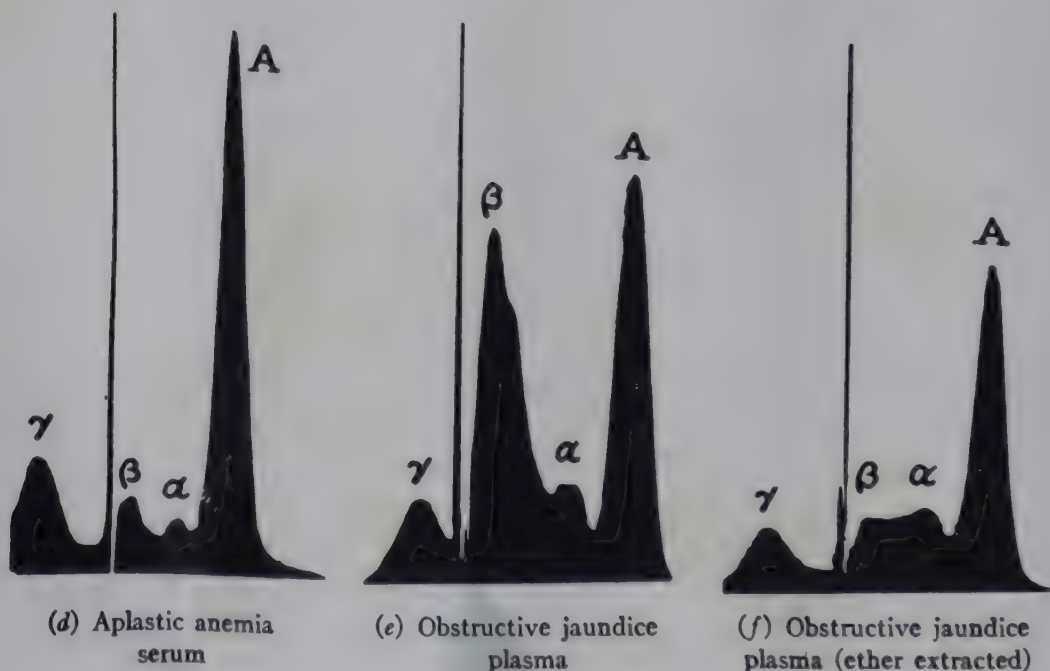
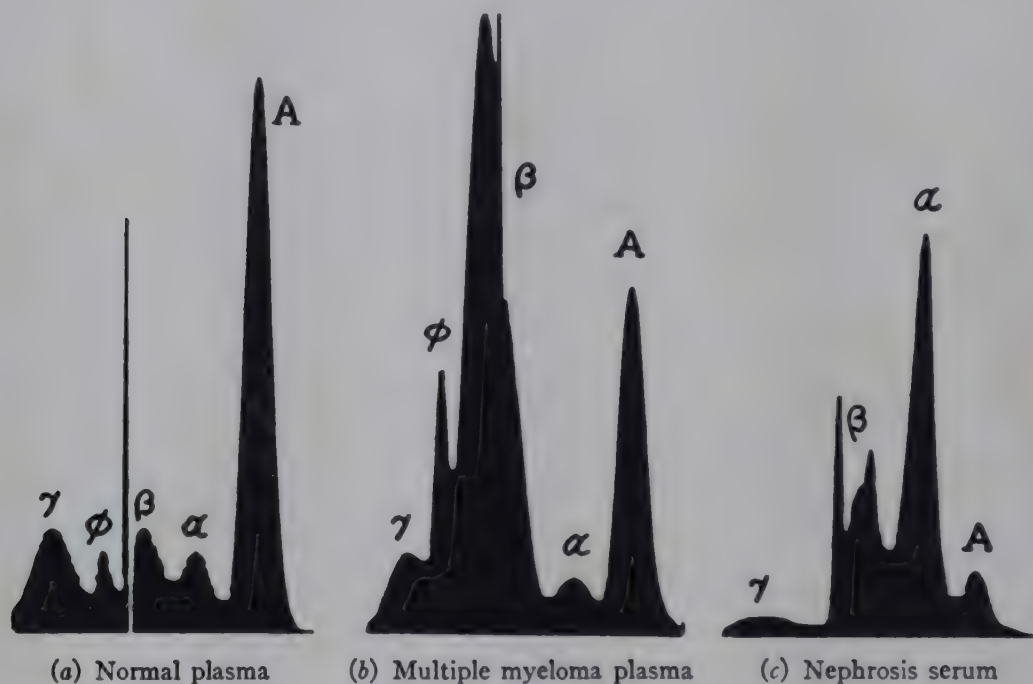


FIG. 12. Electrophoretic patterns of some normal and pathological human blood sera and plasmas. Longworth, L. G., Shedlovsky, T., and MacInnes, D. A.: *J. Exper. Med.*, 70:399 (1939).

of pH would therefore locate accurately the isoelectric point of the protein. Table II lists the isoelectric point of some proteins as determined by the moving boundary method. It should be mentioned that the isoelectric point of a protein is also a function of the ionic strength. For instance, Tiselius and Svensson (90) observed a shift of approximately 0.12 unit in the isoelectric point of egg albumin by changing the ionic strength from 0.01 to 0.10.

6. Electrophoretic Analysis of Protein Mixtures

In addition to the application of the moving boundary method in determining mobilities, this method is very useful in furnishing information as to the purity and homogeneity of a protein solution. It can also be employed to separate different components from protein mixtures.¹² One of the important facts revealed by this method is that serum globulin is heterogeneous electrophoretically (85). The presence of a number of distinctly different components in the globulin is further established by their isolation, using electrophoretic separation (86). This has led to an extensive study of human serum or plasma in normal and pathological conditions. The results indicate a marked change in the concentrations of albumin and globulin components in pathological specimens. Figure 12 shows the electrophoretic patterns of some pathological sera and plasmas investigated by Longsworth, Shedlovsky and MacInnes (38). It may be noted that there are peaks for albumin, A, the three globulins, α , γ and β , and fibrinogen, ϕ . The most striking change is in the β -globulin of the multiple myeloma plasma.

a. Electrophoretic separation

Consider that a protein mixture containing three components, A, B, and C with the different mobilities $u_A > u_B > u_C$, is put into the cell as shown in Fig. 13a. Before the onset of the electric current, the lower middle section is moved to the right to form a sharp boundary between the protein solution and the buffer. After a period of electrolysis, component A migrates ahead from the main body of the protein solution in the upper cathode section, while the slowest moving component, C, is left behind in the lower anode section. We can therefore observe three boundaries in both limbs of the cell, as is indicated in Fig. 13b. If the experiment is continued component A would have moved out of the U-tube and C into the bottom section before actual separation has been accomplished.

¹² For biological and medical applications of electrophoresis, see a review by Luetscher (51a).

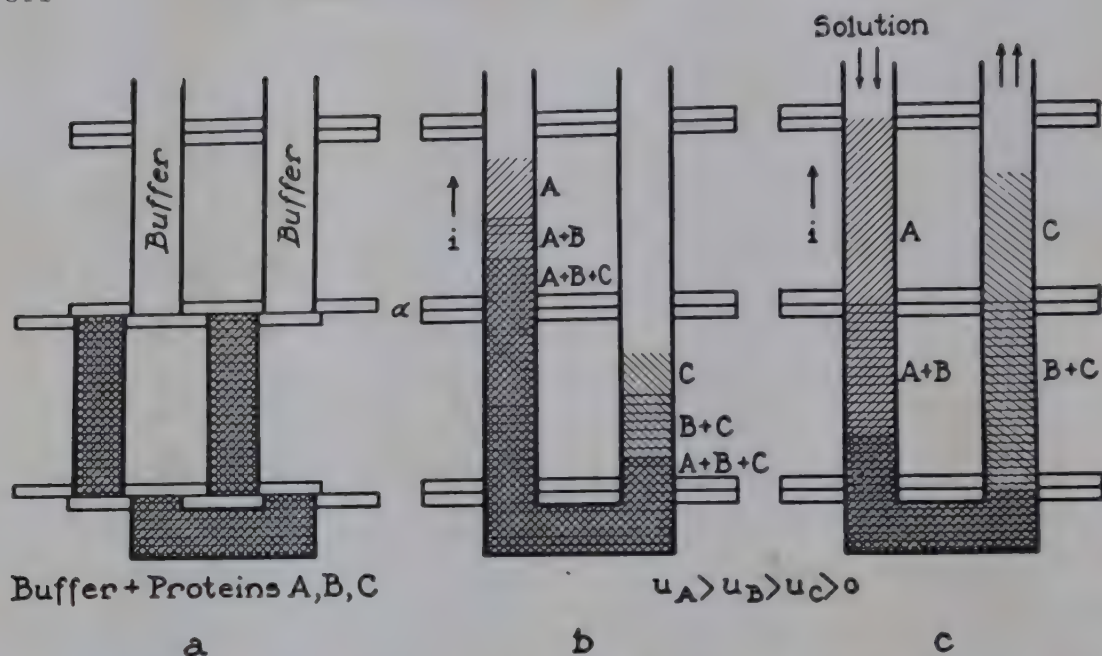


FIG. 13. Ideal electrophoresis of a protein mixture. Longworth, L. G., and MacInnes, D. A.: *Chem. Revs.*, 24:271 (1939).

By application of the compensation device it is possible to adjust the mobilities of the three components in such a way that C would have an apparent negative velocity. As illustrated in Fig. 13c, pure A and C could be recovered in the upper arms of the cell.

Before the recovery of the pure components, their concentrations could be estimated from the scanning pattern. It is evident from equation V, that the area under each peak in the pattern is proportional to the concentration of that component in the mixture. In determining the area due to a given component with a planimeter, it is necessary to make a more or less arbitrary separation due to the fact that the refractions of different boundaries overlap. Tiselius and Kabat (88) suggest that the areas be divided by vertical lines drawn from the lowest point between two adjacent peaks, as is shown in Fig. 14. If the specific refractive increment, k , of a component is known, the concentration of that component can be computed by equation V. Since the other quantities¹³ in equation V are known with ample precision, the concentration thus calculated should be in good agreement with those obtained in the usual manner from Kjeldahl nitrogen determinations. This has been shown to be the case (44, 42). On the other hand, if data on the specific refractive increment are not available, the area under the peak in the electrophoretic pattern could be used for the estimation of the relative concentration of that particular component.

¹³ For the determination of quantities a and b, see reference (45).

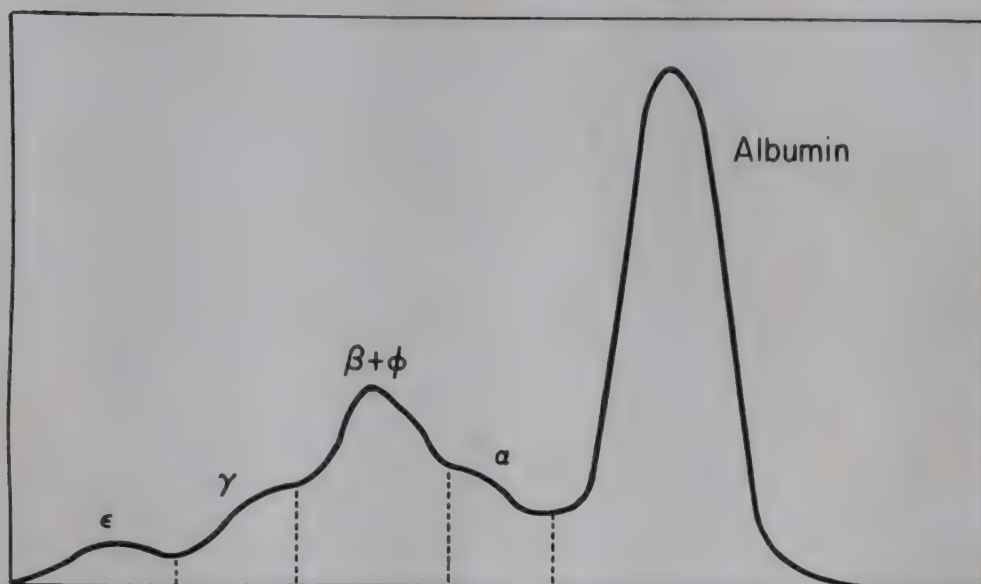


FIG. 14. Tracing of an electrophoretic pattern of hypophysectomized rat plasma illustrating the distribution of the areas among the components.

b. Electrophoretic homogeneity

It is apparent from the foregoing discussions, that a protein mixture containing n components may give rise to n boundaries in the cell upon passage of an electric current. Thus, a pure protein would appear as a single boundary in the electrophoretic pattern. However, it is not always true that one peak in the scanning photograph indicates a singularity of the protein in solution. For instance, lactogenic hormone derived from sheep pituitaries and that prepared from ox glands are distinguishable by their tyrosine content (49) and solubility (48) but they are identical in electrophoretic behavior (47). Moreover, a protein solution which gives only one boundary in a buffer of certain pH may contain more than one component in another buffer of different pH. In Fig. 15a a pattern of the rising boundary from a crystalline β -lactoglobulin preparation in acetate buffer of pH 5.6 is shown. The single peak indicates the preparation to be electrophoretically homogeneous. When the same preparation was electrolyzed at pH 6.5, it appeared to consist of at least two components (50a) as shown in Fig. 15b.

Leutscher (51) reports that some crystalline serum albumins have two boundaries at pH 4.0 but not at pH 7.4. Therefore, it is essential to examine the electrophoretic behavior of a protein within its stability zone in as many buffers of different pH as possible. In general, two or three experiments at different pHs on both sides of the isoelectric point may be sufficient to establish the electrophoretic homogeneity of a protein. As has already been mentioned,

the protein concentration for homogeneity studies should not be less than 1%, for the electrophoretic method is only capable of detecting a protein impurity at a concentration of about two-tenths of a percent if its mobility is different from that of the main component.

While a protein may behave as a single component in electrophoretic experiments, it may still be said to have electrophoretic inhomogeneity if it shows the phenomenon of "reversible boundary spreading." This phenomenon was first observed by Tiselius and

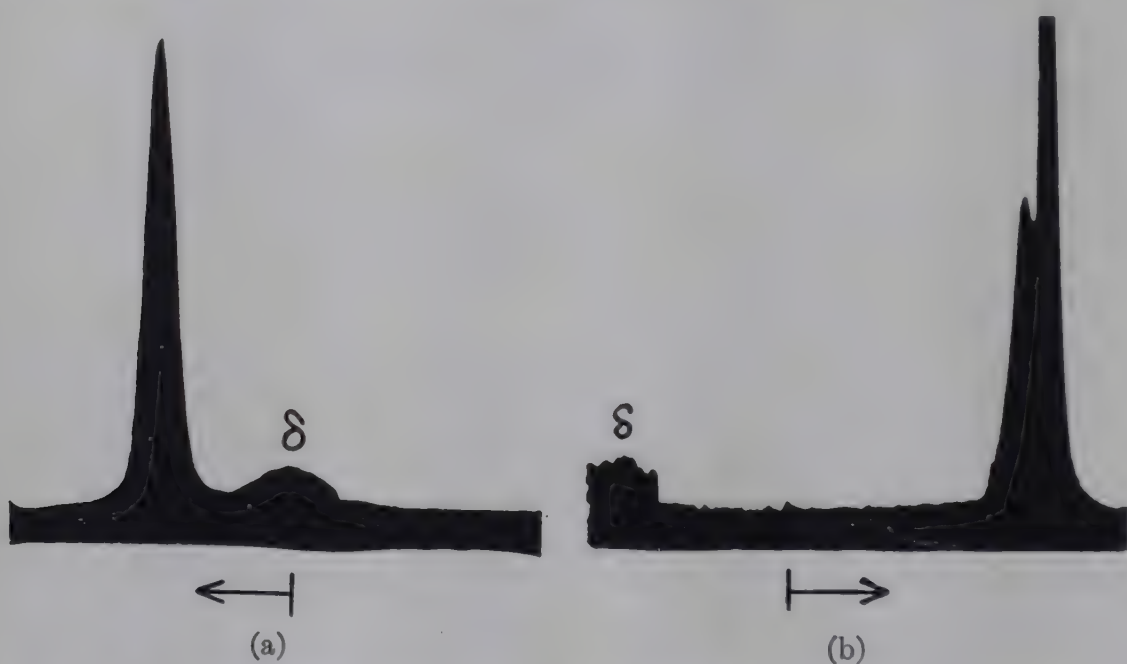


FIG. 15. Electrophoretic patterns of a crystalline β -lactoglobulin preparation. (a) pH 5.6 acetate buffer of ionic strength 0.10 and (b) the same preparation at pH 6.5 acetate buffer of same ionic strength.

Horsfall (87) and was attributed either to the fact that "the proteins exist in a number of interchangeable forms, closely related electrochemically and in a state of equilibrium with each other or that they exist in a large number of stable forms which differ but slightly from each other electrochemically." Proteins such as hemocyanin (87), ovomucoid (39), rabbit papilloma virus protein (81), horse pseudoglobulin (82) and bovine globulins (27a) show the reversible boundary spreading phenomena. In these cases, "an initial sharp boundary will become diffuse as the electrolysis proceeds but if the direction of the current is reversed it will progressively recover most of its initial sharpness" (39). Sharp and co-workers (83) have derived an equation to express this phenomenon based on the distribution of mobilities among the protein ions.

The spreading of the protein boundary was further investigated by Alberty, Anderson and Williams (2a) and their results show that reversible spreading indicates heterogeneity.

If a heterogeneous protein has a Gaussian distribution of mobilities, the heterogeneity constant h may be calculated by Alberty's equation (2b):

$$D^* = D + \frac{E^2 h^2}{2} t_E \quad (\text{XXIIIa})$$

where D^* is the "apparent diffusion constant" calculated from the experimental curves during the electrophoresis, E the electric field strength, t_E time after application of the electric field, and D the normal diffusion constant at zero time. According to the equation, the plot of D^* against time of electrophoresis should be a straight line with a slope $E^2 h^2 / 2$ from which the heterogeneity constant h may be computed. In order to obtain this constant it is essential that electrophoresis spreading experiments should be carried out under conditions such that no convection is caused by the conductivity, pH, and thermal effects and by electro-osmosis. To satisfy these conditions, the experiment is generally performed as close to the average isoelectric point of the protein as possible and at a low protein concentration. Using this procedure, Alberty, *et al.* (2a) found that immune lactoglobulin, human γ_2 -globulin, β -lactoglobulin, and ovalbumin gave h values that varied from 0.2 to 0.66×10^{-5} cm.² per sec. per volt, indicating that these proteins are inhomogeneous. On the other hand, crystalline ribonuclease was found to be completely homogeneous by the electrophoretic criteria.

VI. SOLUBILITY

1. General Considerations

The effect of salt on the solubility of proteins has long been employed for the separation of proteins from one another. Some proteins are very soluble in water but insoluble in the presence of salt, while others are insoluble in water but are soluble at low salt concentration and are precipitated out of solution by high salt content. Such solubility characteristics depend not only upon the intrinsic nature of the protein but also on the type of salt employed. As early as 1905, Mellanby (57) showed that the solvent action of a neutral salt is proportional to the square of the valency of its ions. Cohn (9) later generalized the solubility of proteins in con-

centrated salt solution by the equation

$$\log S = \beta - K_s \mu \quad (\text{XXIV})$$

where S is the solubility of the protein in gm. per liter, μ the ionic strength, K_s the salting-out constant characteristic of the protein and the salt, and β the intercept constant. In general K_s is independent both of temperature and pH, whereas the variation of β

TABLE III
SOLUBILITIES OF SOME PROTEINS IN WATER

Protein	Temperature Degrees	Solubility	Reference
		gm./liter	
Zein	25	0.054	(1)
Casein	25	0.11	(2, 3)
	5	0.043	
Lactoglobulin	25	0.58	(4)
	5	0.35	
Oxyhemoglobin	0	11.2	(5)
Prolactin (ox)	7	0.102	(6)
Insulin	5	0.009	(7)

(1) Cohn, E. J., Berggren, R. E. L., and Hendry, J. L.: *J. Gen. Physiol.*, 7:81 (1924).

(2) Pertzoff, V.: *J. Gen. Physiol.*, 10:961 (1927).

(3) Cohn, E. J.: *J. Gen. Physiol.*, 4:697 (1922).

(4) Cohn, E. J., Ferry, J. D., and Blanchard, M. H.: unpublished, quoted by Cohn, E. J., and Edsall, J. T., "Proteins, Amino Acids and Peptides," Reinhold Corp., New York, 1943, p. 587.

(5) Cohn, E. J., and Prentiss, A. M.: *J. Gen. Physiol.*, 8:619 (1927).

(6) Li, C. H., Lyons, W. R., and Evans, H. M.: *J. Gen. Physiol.*, 24:303 (1941).

(7) Cohn, E. J., Ferry, J. D., Livingood, J. J., and Blanchard, M. H.: *J. Am. Chem. Soc.*, 63:17 (1941); *Science*, 90:183 (1939).

with pH and temperature is very marked. Thus, the value of K_s for horse hemoglobin (17) in ammonium sulfate is 0.71 and that for fibrinogen (14) 1.46. If phosphate buffer is used as precipitating agent, the salting-out constant for horse hemoglobin becomes 1.00 and fibrinogen 2.16. The intercept constant, β , is the logarithm of the hypothetical solubility of the protein at zero salt concentration; it reflects the physicochemical state of the protein. In Table III, the solubilities of some proteins in water are listed. The extremely low solubility of insulin may indicate that it has a low dissociation constant and possibly a small number of free polar groups in the molecule.

It is generally observed that the minimum solubility of a protein occurs in the region of the pH of its isoelectric point. On either side of the isoelectric point, the protein becomes more soluble by the addition of acid or base. By assuming that the protein carries a divalent charge, Green (18) described the solubility of a protein at different pH's by the equation:

$$\frac{S}{^{++}\text{Protein}^{--}} = \frac{S}{S_0} = 1 + \frac{\alpha_H^2}{K_1 K_2} + \frac{K_3 K_4}{\alpha_H^2} \quad (\text{XXV})$$

in which S_0 is the solubility of the neutral molecule, α_{H^+} the activity of the hydrogen ions, K_1 and K_2 the dissociation constants in acid solution and K_3 and K_4 the dissociation constants in alkaline solution. This equation has been found to be in accordance with the solubility data of egg albumin, hemoglobin and casein. In the case of lactoglobulin, Grönwall (19) could not verify the validity of equation XXV, but his data satisfied equation XXVI as derived by Linderstrøm-Lang (46).

$$\log \frac{S}{S_0} = 2.5(\text{pH} - \text{pH}_0) \quad (\text{XXVI})$$

where pH_0 is the isoelectric point and S and S_0 are the solubilities at pH and pH_0 respectively.

In a comprehensive theoretical treatment, Scatchard and Kirkwood (67) and Kirkwood (30) have shown that the negative logarithm of the activity coefficient of an isoelectric dipolar ion is directly proportional to the ionic strength and inversely proportional to the square of the dielectric constant of the solution:

$$-\ln \gamma = \frac{C\kappa^2}{2DkT} \quad (\text{XXVII})$$

Here γ is the activity coefficient of the isoelectric dipolar ion, which for sparingly soluble proteins is equal to the logarithm of the quotient S/S_0 , where S is the solubility at the ionic strength in question and S_0 the solubility at zero ionic strength; D is the dielectric constant of the solvent; k , the Boltzmann constant; T , the absolute temperature; κ is the Debye-Hückel parameter (see equation XVIII); C is a constant derived from the configuration of charges on the surface of the molecule. When this equation is compared with equation XXIV, their identity becomes apparent. Grönwall (19) showed that his data together with Palmer's for β -lactoglobulin (62) agree with the requirements of equation XXVII so long as the ionic strength is lower than 0.020; at higher ionic strength, the plot of the logarithm of the solubility versus the ionic strength begins to deviate from a linear course.

According to equation XXVII, which predicts that the logarithm of the solubility is a linear function of the reciprocal of the dielectric constant, it may be expected that the solubility of a protein should increase with increasing dielectric constant of the solvent. This was found to be the case by the investigations of Richards (65), Ferry (13), and Grönwall (20), who found that the solubility of carboxyhemoglobin and lactoglobulin is increased in the presence

of glycine. The solubility-increasing effect of glycine was shown to be a function of the increase in the dielectric constant. Similar results (20, 21, 22) of other dipolar ions on the solubility of isoelectric proteins have also been obtained.

The influence of temperature on the solubility of proteins depends upon the medium in which the protein is dissolved. Under certain conditions, the protein tends to precipitate out of solution at higher temperature; the solubility of the same protein may increase with temperature when it is dissolved in a different solvent. For instance, horse carboxyhemoglobin (17) has a solubility of 22.7 gms. per liter at 0° in phosphate buffer (1.267 *M*, pH 6.6), while at 25° the solubility is 2.58 gm. per liter. This effect of temperature on the solubility of horse carboxyhemoglobin is reversed at low concentrations of salt. Sørensen and Höyrup (70) found a relatively slight influence of temperature on the solubility of egg albumin. Thus far, there are no mathematical formulations applying to the relationship between temperature and solubility of proteins.

Solubility studies are not only useful in the fractionation of protein mixtures but are also valuable in characterizing protein molecules. Thus, Landsteiner and Heidelberger (35) found that the hemoglobins of some species are different in solubility but indistinguishable serologically and ultracentrifugally. Prolactins isolated from ox and sheep pituitaries have identical electrophoretic (47) and immunological behavior (4); they can, however, be differentiated by their solubility characteristics (48).

Early studies of Hardy (25) and Mellanby (57) have shown that the solubility of serum globulin depends upon the ratio of the amount of saturating body to solvent volume. Similar phenomena have also been revealed by Sørensen and his collaborators in studies with serum proteins (71), egg albumin (70) and casein (72). These experiments led Sørensen to propose the theory of "reversibly dissociating systems" to explain the solubility anomalies, which he considered to be inherent in the nature of proteins themselves. Later Sørensen and Sørensen (73) in a study of the solubility of horse hemoglobin showed that this protein appeared to have a very small "dissociation tendency" and to behave very nearly as a single substance. The fundamental concept that was employed by Sørensen to define singularity of a protein is based on the principles of the phase rule. The work of Northrop and his collaborators (60) on crystalline enzymes has definitely shown that proteins could be prepared which fulfill the requirements of the phase rule as a pure single component.

2. The Phase Rule

a. The phase rule of Willard Gibbs

When water is in equilibrium with its vapor, the vapor pressure increases as the temperature is raised. If a certain temperature is maintained, the vapor pressure of water becomes fixed. Here, we have two phases, liquid and gas and one component, water. In order to maintain the coexistence of water and its vapor, only one temperature is possible at a fixed pressure; *i.e.*, pressure and temperature cannot be varied independently while the number of phases is kept constant. If ice, water, and vapor are in equilibrium, and if we keep the pressure constant and raise the temperature, the ice and water would change into vapor; conversely, if, at constant temperature, we alter the pressure, there will be no longer three phases (solid/liquid/vapor) coexisting in equilibrium. Thus, such a system is rigidly fixed and neither temperature nor pressure could be changed without disturbing the equilibrium of the system.

From the number of coexisting phases and components, Gibbs was able to define the conditions of equilibrium by his celebrated phase rule. This rule¹⁴ may be given by the expression:

$$F = C - P + 2 \quad (\text{XXVIII})$$

in which F represents the degree of freedom of the system, C , the number of components of the system and P the number of phases present. For the application of equation XXVIII, each term in the equation must be specifically defined. Briefly phases are homogeneous parts of a system mechanically separated from the other parts of the system; the number of components is the minimum number of molecular species in terms of which the composition of all phases may be quantitatively expressed and the degree of freedom of a system is the number of variables which must be fixed to define the system. In general, a phase is either gaseous, liquid, or solid and the degree of freedom is one of three independently variable factors, temperature, pressure, and concentration.

b. Application of the phase rule to protein solutions

When a pure protein is brought into contact with a liquid in which it dissolves, a certain amount of it goes into solution and the process continues until the protein concentration reaches a certain definite value. The concentration becomes constant even in the presence of an excess of the solid phase. The solution is said to be

¹⁴ For a full discussion of the phase rule, see (15).

saturated with the protein when a condition of equilibrium has been established between the solid and the solution. By equation XXVIII we have $C=2$ and $P=2$; thus the system has two degrees of freedom. If both pressure and temperature are maintained constant, as they usually are for such determinations, the state of the system becomes rigidly fixed.

In practice, the liquid employed for dissolving the protein is a buffer which contains at least three components: viz., water, salt

TABLE IV
POSSIBLE SYSTEMS FORMED FROM ONE OR TWO PROTEINS
AS OBTAINED FROM THE PHASE RULE

Components	Phases	Degrees of Freedom at Constant Temperature, Pressure, and Concentrations of Acid and Salt	Examples
4 = water, salt, acid protein	1	1	A homogeneous solution.
	2	0	A solid phase plus saturated solution.
	3	(-1)	Two solid phases plus saturated solution. (Only possible at a particular concentration of acid or salt.)
5 = water, salt, acid; two proteins	1	2	A homogeneous solution.
	2	1	One solid phase containing (a) a single protein, or (b) a solution of two proteins, in a solution of variable composition.
	3	0	Two solid phases in solution of constant composition.

(From Butler, J. A. V.: *J. Gen. Physiol.*, 24:189 (1940).)

and acid. Such a system has four components including the protein, two phases and, hence, four degrees of freedom as computed by equation XXVIII. The four degrees of freedom are temperature, pressure, and concentrations of salt and acid. If these variables are fixed, the system is said to be invariant. Therefore, with a saturated solution of a single protein, the concentration is fixed no matter how much solid phase is present. If the protein solution contains two distinct protein components in addition to the buffer, the system has five degrees of freedom according to the phase rule. Since the temperature, pressure, and concentrations of salt and acid are maintained unchanged, the remaining one variable factor is the concentration of one of the protein components. Sometimes

two protein components may form a solid solution (6); the composition of the solution, then, will depend on the composition of the solid solution. If the composition of the solid solution is fixed, the composition of the solution in equilibrium with it is also fixed; the system appears as if it contained one protein component. On the other hand, if the ratio of the protein in the solution is different from that in the solid solution, the concentration of the proteins in the solution will then vary with the amount of the solid, as shown by Northrop and Kunitz (59). Butler (6) has listed the characteristics of the possible systems which may be formed from one or two proteins together with water, acid and salt; these are given in Table IV.

3. Solubility Curves

a. A single pure protein

To a unit volume of a solvent, increasing quantities of a single pure protein are gradually added. At the beginning, the protein is completely dissolved and the solution is homogeneous. As soon as a solid phase begins to appear, the solution is saturated with the protein. Thus, further additions of solids do not cause an alteration of the protein concentration in the solution. If the data is plotted, using the added amount of solid as abscissa and the amount dissolved as ordinate, we have a straight line with *unit* slope before the formation of a saturated solution and another straight line with *zero* slope after the first appearance of a solid phase. Figure 16 presents three solubility curves obtained in three different solvents with a growth hormone preparation isolated from ox anterior pituitary (50) and indicates the type of solubility curve characteristic of a single pure protein.

b. A mixture of two or three proteins

Let us assume that a protein preparation contains two components A and B having solubilities $S_B > S_A$. When this protein preparation is added in increasing quantities to a definite volume of buffer, the solution is first clear and then becomes turbid. The appearance of turbidity is due to the insolubility of A. The concentration of proteins in the solution is, however, not yet constant, for B is still soluble. It is assumed that the solubility of A is not influenced by the presence of B and vice versa. When more solid is added, a point is reached where the solution is saturated with B. At this point, the protein concentration will no longer vary. Thus,

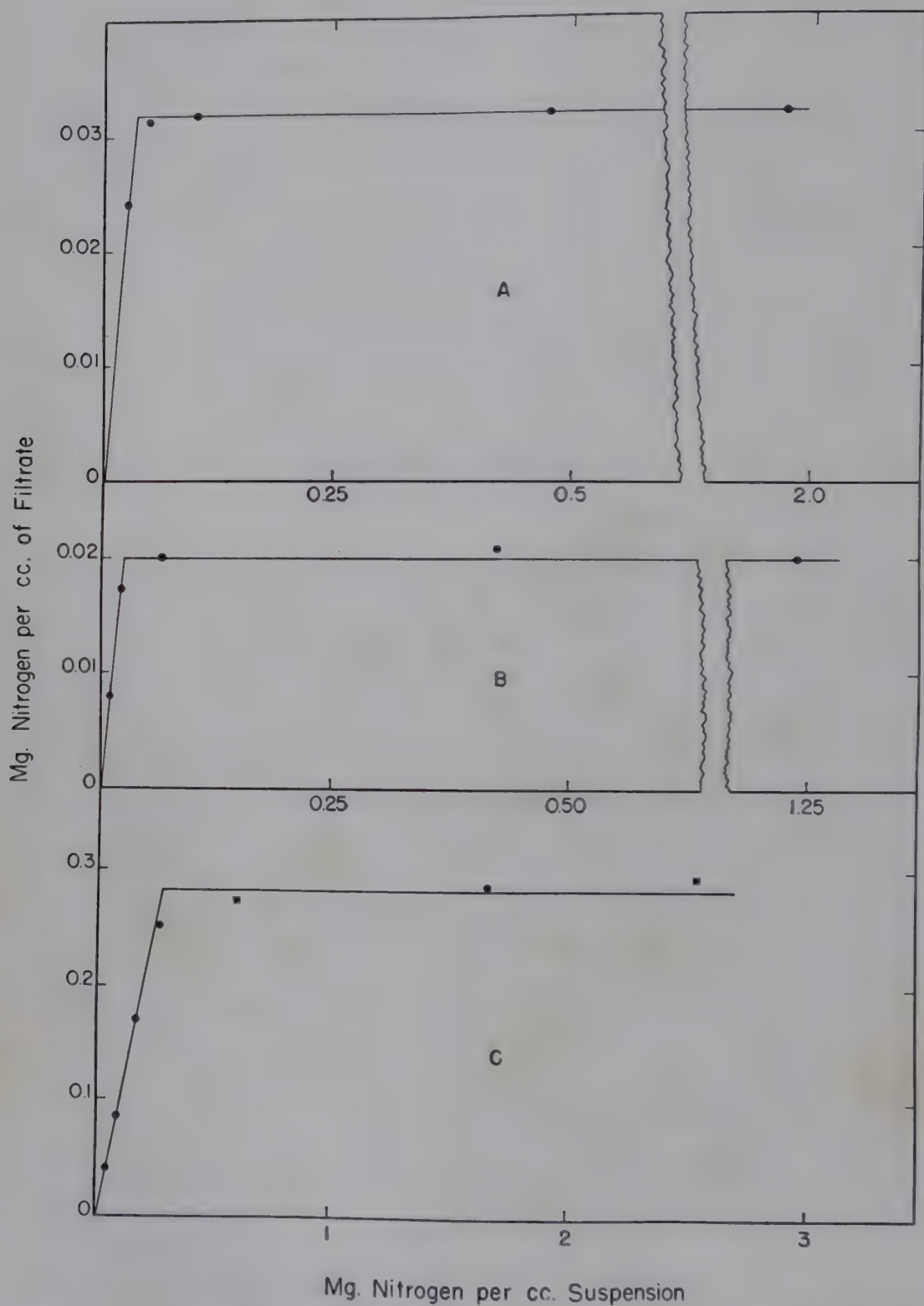


FIG. 16. Solubility curves of the growth hormone in various solvents at 5° illustrating the singularity of the protein (A) distilled water, pH 7.1; (B) 4.8 M NaCl in 0.07 M phosphate buffer, pH 5.7 and (C) 3.8 M NaCl in 0.07 M phosphate buffer, pH 6.4. Li, C. H., Evans, H. M., and Simpson, M. E.: *J. Biol. Chem.*, 159:353 (1945).

there are two sharp breaks in the solubility curve of this system, indicating the presence of two components in the protein preparation. Similarly, if the mixture has three proteins, three sharp breaks would be observed in the solubility curve. Northrop and Kunitz (31, 32, 59) have analyzed solubility curves of this type and have shown that the composition of the protein mixture can be computed from its curve. Figure 17 shows a solubility curve for a

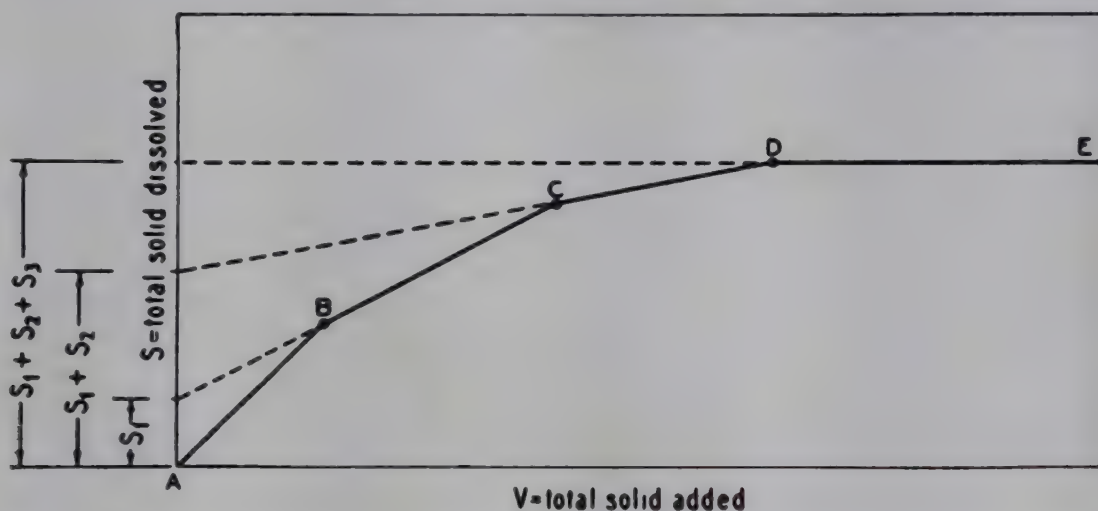


FIG. 17. Theoretical solubility curve for a system of four components consisting of one liquid and one, two or three solid phases. The total solid added is plotted against the amount of solid in solution. Kunitz, M., and Northrop, J. H.: *Cold Spring Harbor Symposium Quant. Biol.* 6:325 (1938).

mixture of three protein components. The following analysis is essentially the same as that given by Kunitz and Northrop (32).

From points A to B in the solubility curve, the solution is homogeneous and contains no solid phase; the amount of solid dissolved, S , is equal to $P_1V + P_2V + P_3V$ where V is the total solid added to the system and P_1, P_2, P_3 the fraction of each component in the solid added. All concentrations are expressed in gm. per ml. At B, where $P_1V_B = S_1$, S_1 being the solubility of component 1, a solid phase of component 1 appears. Hence, from B to C the, only solid phase present is component 1. The equation of line BC is

$$S = S_1 + V(P_2 + P_3)$$

S_1 is the intercept and $(P_2 + P_3)$ is the slope of line BC. At C, $P_2V_C = S_2$, and component 2 appears as a solid phase. From C to D, the concentrations of proteins becomes:

$$S = (S_1 + S_2) + P_3V$$

$(S_1 + S_2)$ is the intercept, and P_3 is the slope. Hence S_2 is the inter-

cept of CD minus the intercept of BC. At D, $P_3V_D = S_3$ and component 3 appears as a solid phase. From D on, the solid phase consists of the three components and the solid dissolved becomes independent of the amount of protein in the system. The equation of line DE, therefore, is

$$S = S_1 + S_2 + S_3.$$

Similar analysis can be applied to any number of components in the system so long as no solid solution is formed.

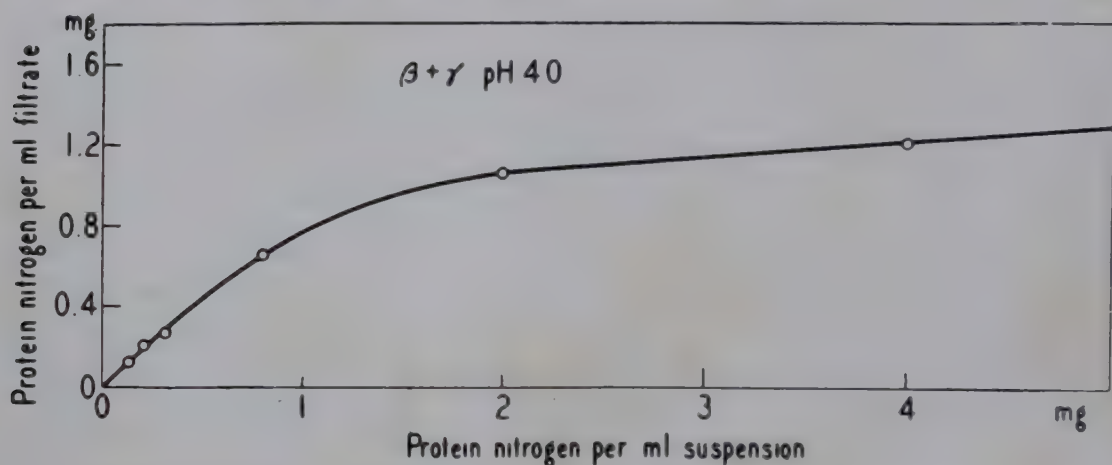


FIG. 18. Solubility of artificial mixture of crystals of β and γ chymotrypsin illustrating the "solid solution" type of curve. Kunitz, M., and Northrop, J. H.: *Cold Spring Harbor Symposium Quant. Biol.* 6:325 (1938).

c. Solid solutions

In the case of solid solutions, there are no breaks in the solubility curve; the initial slope gradually deviates from unity and the curve continues to rise until, in the presence of great excess of the solid, a horizontal line results. Such a plot is illustrated by Fig. 18 taken from the paper of Kunitz and Northrop (32).

In some cases, the solubility curve of a mixture is of the "solid solution" type in one solvent but not in another. For instance, an artificial mixture (32) of 40% β -chymotrypsin and 60% of the γ form, in 0.4 saturated ammonium sulfate at pH 4.0, gives a solubility curve like a solid solution. If the same mixture is dissolved in 0.4 saturated ammonium sulfate at pH 5.5, instead of pH 4.0, the curve is like that of a true mixture of two proteins.

It is clear that if solubility curves are to be used to establish the purity of a protein, they must be obtained in more than one solvent. In each solvent, most of the solubility data should be obtained in the region where the solid phase begins to appear, in order that the differentiation of a true mixture from a solid solution

can be judged with certainty. If the material has constant solubility in several different solvents, it can be taken as a strong indication that the material is a single pure protein.

4. Practical Procedure

a. Necessary precautions

Solubility experiments should be carried out either in a constant temperature room or in a thermostat. Since proteins are generally denatured or deteriorate more quickly at higher temperatures, it is preferable to conduct the experiments at a low temperature (0–10°). If the solution contains organic solvents, even lower temperatures may be necessary.

The choice of a solvent depends on the type of protein to be studied. In general, a solvent with good buffering action is used; it should have no denaturing effect on the protein and its solvent action should not be too high or too low. It is best to have a solvent which will dissolve enough of the protein so that the analytical technique for measuring the amount dissolved can be sufficiently accurate.

There are many ways to agitate the solution. It is important that foaming be avoided during agitation, as it is well known that foaming denatures proteins and that denatured proteins have lower solubilities. The following methods of agitation are generally used: (a) the test tubes containing the solid and solvent are clamped in a horizontal position on the outer diameter of a revolving wheel driven by a slow-moving motor; (b) the test tubes are attached to a rocking device; and (c) the solid and solvent in a test tube are stirred by means of a glass screw stirrer as described by Findlay (16).

If the phase rule is to be applied, it is necessary to ascertain that the system is in equilibrium. In general practice, if the same solubility values of the solid are obtained either from supersaturated or from undersaturated solutions, it can be certain that a true equilibrium has been established. The approach to equilibrium from supersaturation may be achieved by two ways: (a) by mixing solvent and solute at a higher temperature and then cooling to the temperature of the measurement, and (b) by dissolving the solid in solvent A and then mixing with solvent B to cause the solid to precipitate. The author has found that method b is suitable for solubility measurements of amorphous preparations. The time required to attain equilibrium must be determined experimentally in each case studied.

b. Experimental technique¹⁵

Before conducting the solubility determination, the solid must be washed two or three times with the solvent or dialyzed against the solvent for at least 24 hours. The solid is then stirred or precipitated with successive portions of the solvent until the same quantity of dissolved solid is found in the last two or three aliquots of the solvent. Varying amounts of this suspension are distributed in a number of test tubes (12×100 mm. Pyrex) and each tube, filled with the solvent, contains a glass bead or ball-bearing. Most of the tubes should contain a small excess of solid rather than a large excess. The tubes are then stoppered with one hole rubber stoppers and the last bubble of air removed by plugging the hole with a glass rod. The tubes are next agitated on the wheel for two or three days until true equilibrium is reached. The solutions are either filtered through ashless filter paper (Whatman No. 42) or centrifuged. If centrifugation is employed, all test tubes should be centrifuged together in the same machine at the same time. If filtration is employed, the first portion of the filtrate should be discarded. The filtrates or supernatants are then analyzed for the amount of dissolved solid.

c. Determination of dissolved protein

Several methods have been employed to determine the quantity of dissolved protein. Kjeldahl nitrogen analysis is most commonly used. If the solvent contains salts with a high nitrogen content, turbidity (31) or the tyrosine color value (48) may be employed for protein nitrogen estimation.

The turbidity method of Kunitz and Northrop (31) may be outlined as follows: a 5 ml. sample is mixed in a 150×15 mm. test tube with 5 ml. of 5% trichloroacetic acid made up in 0.25 saturated ammonium sulfate. The mixture is allowed to stand at least five minutes at 20° and then placed for 10 minutes into an 85° water bath. The test tube is immersed in the hot water to a depth of about 2–5 mm. above the level of the liquid inside the tube and is loosely stoppered during the heating. The tube is stoppered tightly immediately after removal from the bath and allowed to cool to room temperature. The turbidity of the well mixed suspension is determined in a photoelectric colorimeter. The protein nitrogen is read from a calibration curve for the turbidity of several concentrations of the standard solution.

¹⁵ See discussions given by Herriott (27).

The phenol color value technique is carried out in the following manner. To 1 ml. of the filtrate, diluted to 10 ml., are added 2 ml. of 1 *M* NaOH and 3 ml. 1:3 Folin's reagent. The mixture is kept in an oven or a water bath at 40° for 15 minutes. The color produced is measured in a photoelectric colorimeter using a red filter. The protein content is then read from a standard curve which has been constructed by using known quantities of protein in the same manner.

If the protein is biologically active, the amount of dissolved solid can be determined by a bio-assay technique. For instance, Northrop (60) employed peptic activity to plot his solubility curves and found that they were identical with those obtained by nitrogen determination. Since most bioassays are not sufficiently accurate, they should not be used alone to obtain data for plotting a solubility curve. However, bioassay is very useful in determining whether the soluble and insoluble solid have been fractionated in their biological potency.

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Chapter IX

CHEMICAL REACTIONS OF PROTEINS

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I. INTRODUCTION


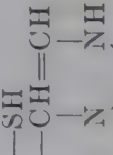

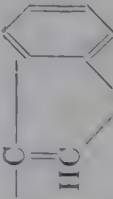
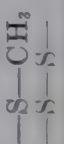
AT THE turn of this century, on the foundation of Emil Fischer's work on peptides, organic chemists began to apply their skill and their techniques to the study of intact proteins. This early work suffered from severe handicaps. Thus, there were not many proteins of established purity available, and knowledge of their composition was quite incomplete. Such uncertainties gradually tended to deter many organic chemists, wary from the start of tackling these "colloidal" monster molecules. A change in this attitude began to come about with the isolation of crystalline urease, insulin, and pepsin, and chemical work on these and other proteins has increased steadily in the past two decades. This development was favored by the simultaneous wave of interest in high polymer chemistry. Organic chemists have learned to get along without melting points, and to rely on physicochemical techniques, besides their old and newly developed analytical and synthetic methods. With advance in knowledge, proteins have lost part of their aura as mysterious components of living matter, and have come to be regarded as complex polymeric chemicals of approximately known constitution. As such, they may be subjected to a variety of reactions involving one or several of their different types of reactive groups.

The reactive groups of proteins are primarily the active hydrogen atoms of the side chains of the dicarboxylic and dibasic amino acids, of tyrosine, cysteine, serine, threonine, hydroxyproline, glutamine, and asparagine, as well as of the terminal amino and carboxyl groups of the peptide chains. To these have to be added the reactive $-\text{CH}$ and $>\text{NH}$ groups on the aromatic and heterocyclic

\parallel
rings of tyrosine, tryptophan, and histidine; the disulfide bonds of cystine; the thioether bonds of methionine (Table I). The peptide bonds might also be listed except for the fact that they appear to be generally rather inert to all but hydrolytic agents which cause degradation of the protein molecule and thus are not part of the subject of this chapter.

Nine classes of protein groups carry reactive hydrogen atoms. Of these, as listed in Table I, the first three are acidic, the next three are basic in nature, and the rest carry no charge under normal conditions. The total number of the different types of groups varies greatly from one protein to another. Of the approximately 90 amino acid residues (moles) per 10^4 g. of an average protein, as many as 37 may carry aliphatic hydroxyl groups, as in silk sericin, or 32

TABLE I
REACTIVE GROUPS OF PROTEINS

Group ^b	Structure	Origin	Typical Proteins Relative Abundance ^a						Proteins or Polypeptides of Extreme Composition	
			β -Lacto- globulin	Egg Albumin	Insulin	Gliadin	Bovine Serum Albumin	Edestin	Substance	Relative Abun- dance ^a
Carboxyl	—COOH	Glutamic acid Aspartic acid Peptide chain end	14.5	10.7	9.8	3.7	12.6	8.4	Polyglutamic acid	78
Phenol		Tyrosine	2.1	2.2	7.0	1.8	3.0	2.4		
Sulphydryl Imidazole,		Cysteine Histidine	0.6 1.0	1.0 1.5	0 3.3	0 1.2	0.3 2.5	0.25 1.9		
Amino	—NH ₂	Lysine	8.9	5.1	5.0	1.0	9.0	1.8	Polylysine	72
Guanidyl		Peptide chain end Arginine	1.7	3.3	1.7	1.6	3.6	9.3	Salmine sulfate	38
Amide	—CONH ₂	Glutamine Asparagine	7.7	7.2	9.8	32.0	6.2	12.7	Polyglutamine	62
Aliphatic hydroxyl	—OH	Serine	8.2	11.1	6.7	6.4	9.7	9.3	Sericin	35
Indole		Threonine ^c Tryptophan	1.0	0.7	0	0.3	0.3	0.7	Gramicidin	20
Thiomethyl Disulfide		Methionine Cysteine	2.2 1.0	3.5 0.4	0 4.9	1.1 1.1	0.5 2.4	1.6 0.4	Hair-Keratin	8

may carry primary amide groups, as in gliadin.¹ The extreme cases found to occur in nature are the protamines in which two out of three residues bear guanidyl groups, and the glutamic acid polymers of the capsular material of certain microorganisms (*B. anthracis*, *B. subtilis*). On the other hand, there are proteins deficient in certain types of amino acid residues, such as zein, which contains almost no amino nitrogen, and gelatin, which contains less than one equivalent of tyrosine, tryptophan, and histidine combined (per 10^4 g.). The availability of such proteins of extreme composition has proven of great help in the elucidation of protein reactions. The approximate occurrence of the various reactive groups in some proteins of average, and in some of extreme composition, are listed in Table I.

The present concept of the structure of a native globular protein is as follows (159): One or several peptide chains, possibly cross-linked through disulfide bonds, are held in an orderly coiled configuration through ionic and hydrogen bonds. The same forces maintain the linear orientation of fibrous proteins. The protein groups responsible for these secondary bonds are, in general, the same which have been listed above as chemically reactive. In addition, the peptide bonds, probably owing to their numerous and spatially regular occurrence, appear to play an important role in hydrogen bonding. Thus, it is evident that chemical modification of an appreciable number of any of the reactive groups may alter the "native" properties of the protein. On the other hand, reactions which affect only the secondary forces in a protein, viz. denatura-

¹ Throughout this chapter the occurrence of groups and residues will be expressed in terms of gram-moles per 10^4 g. of protein. This permits a simple additive treatment of classes of residues (serine and threonine, glutamic and aspartic acid, etc.). It also makes the data independent of uncertainties in the molecular weight of proteins. To transform the data as here presented to a molar basis, they have to be multiplied by 3.5 for M. W. 35,000 or 7 for M. W. 70,000, etc.

^a Gram-equivalents of various groups per 10^4 g. protein.

^b The carboxylic, sulfhydryl, amino, and indole group analyses are based on determinations on the intact proteins. The other groups are calculated from the amino acid composition of hydrolysates. Recent values obtained or listed by Chibnall (35) were used for egg albumin, gliadin, and edestin; Brand's values were used for bovine serum albumin (22); the values of both authors were averaged for insulin and β -lactoglobulin. In the case of egg albumin and β -lactoglobulin, titration curves have given values for guanidine residues in excellent agreement with the arginine contents, but the titrimetric imidazole values did not agree as closely with the well established histidine contents of these proteins. The number of carboxyl groups of β -lactoglobulin, calculated from the dicarboxylic acid minus the amide content of hydrolysates, is 0.4 equivalent (per 10^4 g. protein) lower than the number of carboxyl groups determined titrimetrically (28) or colorimetrically, i.e., total acid minus phenolic and sulfhydryl groups (61, 65). Such differences as well as those between the lysine content and the total amino nitrogen (as determined by titration curves or the Van Slyke method) represent a measure of the terminal groups, and thus of the average length, of the peptide chains. This is illustrated particularly by insulin which contains only 1.7 lysine residues, yet has 5.0 amino nitrogen atoms. In this case the nature of the terminal α -amino groups has been established and thus all doubt concerning their existence removed (202a). The multiple chain concept of insulin is further substantiated by the excess in the number of total carboxyl groups over that derived from dicarboxylic and amide analyses (3.2 equivalents per 10^4 g.).

^c Hydroxyproline is known to occur in appreciable amounts only in gelatin and isinglass (9.5 and 3.4 equivalents, respectively). No reliable method for its determination in small amounts is available, and it is therefore not included in the table. The same is the case for hydroxylysine which occurs to the extent of 1% in gelatin and still less in all other proteins analysed.

tion, may permit the molecule to uncoil, and thus increase the chemical reactivity of many side chains. Such facts have been observed long before the mechanism of denaturation was understood. In discussing protein reactions, brief attention will, therefore, first be paid to those involving only coordinate or ionic bonds. This will be followed by a discussion of the typical protein reagents and reactions affecting primary bonds.

II. REACTIONS INVOLVING ONLY SECONDARY OR IONIC BONDS

1. Denaturation

Denaturation has been variously defined.² Most generally, denaturation appears to be regarded as the sum of all changes involving only coordinate or ionic bonds, which lead to detectable alterations in the molecular structure. It should be recognized that aggregation, which may or may not lead to precipitation or coagulation at the isoelectric point, frequently follows, but is not necessarily a part of denaturation.

Denaturing agents or conditions are such as can disrupt coordinate and ionic, but not primary bonds. The more common denaturing agents include moderate heat, surface forces, ultraviolet light, hydrogen and hydroxyl ions (of sufficiently low concentration not to cause cleavage of peptide, amide, or disulfide, bonds), and certain water-miscible organic solvents and solutes in high concentrations, such as alcohols, urea, detergents, etc. Different proteins differ greatly in their susceptibility to denaturation by different agents. It appears that the molecular structure of some proteins is more dependent upon hydrogen bonds, while others may be mainly salt-linked. Again, others are almost resistant to denaturing agents. As we learn more about the constituent amino acids, chain lengths and crosslinks of individual proteins, their behavior with regard to various denaturing agents may lose its apparent randomness and fit into a predictable pattern. Thus insulin was recently

² The author has doubts concerning the usefulness of the term if it is meant to cover the great variety of well understood chemical modifications. Apart from this too inclusive definition and from some misleading statements concerning the masked state of amino, phenolic, guanidyl, etc., groups, the review of the chemistry of protein denaturation by Neurath, Greenstein, Putnam and Erickson (165) must be valued highly as an authoritative and comprehensive presentation of our knowledge of this subject. The oversimplified picture of the chemical basis of denaturation, as outlined in the present chapter, is largely derived from this and other review articles (164, 218, 143). The physicochemical and thermodynamic aspects of denaturation, as well as a comprehensive review of the extensive bibliography of denaturation, appear beyond the scope of this discussion. The interested reader is referred to the above review articles.

shown (202a, c) to be composed of subunits of a molecular weight of approximately 12,000, each of which consists of four peptide chains and contains six cystine residues. It is evident that the cystine suffices to supply two crosslinking $-S-S-$ bonds between each pair of chains. Thus, the structure of the subunit appears fixed in an orderly globular manner without the need for extensive hydrogen bonding. It is therefore not surprising that insulin may be subjected without damage to a variety of conditions, such as high temperature, acids, or organic solvents which would create havoc with many other proteins.³ In a similar manner, although on less evidence, one might assume that plant globulins, such as edestin, which are very rich in arginine, rely largely upon salt linkages for their molecular architecture, and are, therefore, easily denatured by acids.

Unfortunately, very little appears to be known about the role of various protein groups in contributing to hydrogen bonding. Mirsky and Pauling (159) attributed it largely to the peptide, carboxyl, and amino groups. On the other hand, Crammer and Neuberger (41) believe that the phenolic groups of proteins may supply strong foci for hydrogen bonds. The fact that reduction of disulfide bonds renders most proteins quite insoluble, and was actually shown to cause aggregation in the case of insulin (156) suggests that the resultant sulfhydryl groups may have a strong tendency to hydrogen bonding with some other protein group. The ability of mercaptans to form hydrogen bridges with nitrogenous compounds appears to have been established (83b, 113b).

The criteria of the denatured state are usually listed as a loss in the solubility, crystallizability, and biological activity, and an increase in chemical reactivity of certain groups, and in the digestibility of the protein by enzymes. Each of these effects appeared as a separate mysterious phenomenon until the general nature of the process was recognized. Now they seem evident logical consequences of the unfolding of the peptide chain and subsequent tendency to random structure, and aggregation. The increased reactivity of certain groups represents one of the few quantitative means of gauging the extent of denaturation and therefore must be discussed in some detail in this chapter.

³ Other proteins rich in disulfide crosslinks, e.g., crotoxin, lysozyme, and keratins, are similarly resistant to most denaturing conditions. However, treatment of insulin with acid at elevated temperature causes its precipitation in seemingly denatured form. This singular reaction, and its reversal, was described by du Vigneaud, *et al.* (229) and has more recently been studied in detail by Waugh (233).

2. Masked Protein Groups

The first groups which were observed to appear in proteins upon denaturation were the sulfhydryl (—SH) groups (5). This phenomenon has since been studied quite intensively. It will be discussed below in connection with the various reagents affecting the —SH groups. Here it will suffice to state that a number of proteins containing cysteine do not give most of the expected tests for —SH groups until they have been denatured. This complete masking of a highly reactive group is a remarkable phenomenon. It has stimulated much speculation. According to a recent attractive theory, the sulfur may be bound in primary, though readily reversible, linkage into a thiazolidine ring (138, 16). At present, however, the concept is more generally held that the —SH group occurs as such but is masked by steric inaccessibility within the folds of the native protein molecule. If the sulfhydryl group tends to form strong hydrogen bonds, as postulated above on the basis of the aggregated state of reduced proteins, this complete masking would become more readily understandable. Many organic chemists may regard as unacceptable the concept of hydrogen bonds suppressing the strong reducing nature of mercaptans. However, it must be kept in mind that any two peptide chains in the native state are cross-linked by a great number of hydrogen and other bonds and that any disrupting agent would have to overcome the sum of these bond strengths.

Another group which, in certain proteins becomes detectable only after denaturation, is the disulfide bond (113a, 183, 158). This group, in contrast to others, is part of the backbone rather than of the side chains of the peptide units, and thus its masked state may be less surprising.

The phenolic groups resemble the sulfhydryl in various chemical aspects, and also in their tendency to be of lessened reactivity in some proteins (107c). The masking of the phenolic groups, however, appears in no case to be complete, at least insofar as concerns their iodination or their oxidizability by Folin's phenol reagent. When the chromogenic values of a variety of native proteins were compared with the sum of their tyrosine and tryptophan contents, they were found to range from 42 to 73%. The highest value was found with insulin, and approached those reported for various tyrosine peptides, i.e., 75–89% (224a, 155), thus indicating that few, if any, phenolic groups are masked in insulin. Similar conclusions were drawn by Crammer and Neuberger (41) from the pK

values of the tyrosine residues of this protein, as determined by spectrophotometric measurements. On the other hand, the low chromogenic value observed with chymotrypsinogen (42% of total) indicates that, in this protein, not only the phenolic but also the indole groups must be in part inaccessible.⁴

A recent study of the iodination of serum albumin (132) has demonstrated the gradual unmasking of an increasing number of phenolic groups with increasingly intense denaturation. A similar increase in their reactivity was observed in the case of lysozyme (58a). Evidence for the occurrence of unreactive amino groups and imidazole in certain proteins was presented by Porter (180a, b). Similar findings led Roche and coworkers to the conclusion that the guanidyl groups of proteins might also vary greatly in their reactivity (191a). However, the acid and basic groups of all proteins studied seem to be titratable over the range of pH 2–10 (28), which seems to indicate that any masking must be pH dependent, or must not affect the polarity of these groups.

Quantitative studies of the number of masked groups liberated under various conditions of denaturation, and with different reagents, have demonstrated the existence of an unbroken scale of reactivity for these groups. No analogies from one protein to another are possible. The concept that denaturation is an all-or-none phenomenon has now been largely replaced by one viewing it mainly as a matter of degree. If only a few hydrogen bonds are broken, without ensuing unfolding and molecular aggregation through the newly formed reactive groups, the course of events can often be reversed and the original configuration more or less closely approached. This "reversibility" of denaturation was first investigated in detail by Anson and Mirsky (4) and has more recently been the subject of a series of interesting and critical studies by Neurath and coworkers (as reviewed by Neurath, 165).

3. Effects of Detergents

Little is known about the mechanism of action of some of the most effective denaturing agents. Only the action of detergents, which have proven highly effective denaturing agents, has been

⁴ Anson (3b) found that the tryptophan residue of chymotrypsinogen did not react with iodine under conditions favorable for iodination of the free amino acid. In a study of the ability of acetyltryptophan and indolepropionic acid to react with iodine under similar conditions (172) (pH 3.2 at room temperature) these models, in contrast to free tryptophan, were found resistant to iodination. This thus represents an illustration of the danger of relying on any one model substance, particularly a free amino acid, in the study of reactive protein groups.

elucidated to a certain extent and has thrown some light on the mechanism of denaturation as a whole (182, 143, 218).

Anionic and cationic detergents combine readily with polar protein groups of opposite charge (219). This appears to be a general property of large ions, irrespective of their detergent nature, and is attributed to an increasing affinity of protein groups for ions of increasing molecular weight. Such ionic binding occurs over a wide pH range, and even at a pH where the net charge of the protein is the same as that of the added ions. Precipitation of the complexes may occur under favorable conditions, but only if the protein and detergent carry opposite net charges. The isoelectric point of a protein may be determined by methods based upon this phenomenon (117, 182).

Higher concentrations of detergents than are necessary for stoichiometric combination and precipitation, lead to extensive denaturation, and often to solubilization of the denatured protein. With anionic detergents, such as alkyl benzene sulfonates, complexes containing increasing amounts of detergent are formed with increasing concentration of the latter (145, 166). The denaturing action of detergents is attributed to the tendency of their aliphatic chains to form aggregates. Thus, the first prerequisite appears to be a center of attachment of the denaturing agent to the protein, such as the basic groups in the case of an anionic detergent. The further criterion for a good denaturant of this type is its tendency to form molecular aggregates, rather than coordinate links with hydrocarbon side chains of the protein. Thus, the detergent micelles may be pictured as pushing the peptide chains apart mechanically and thereby disrupting the hydrogen bonds responsible for native protein configuration. The primary mode of attachment of non-polar denaturants, such as urea and alcohols, must differ from that of the ionic detergents, but their main action must also consist in disrupting the hydrogen bonds between peptide chains.

4. Miscellaneous

In contrast to detergents, a variety of compounds have been found to protect certain proteins against denaturation. Such an effect has been observed upon addition of salts of many fatty acids to serum albumin. This reaction was thoroughly studied (7, 21, 223c) and it now appears that caprylate and similar ions combine with the basic groups of this protein, as do the anionic detergents. But the hydrocarbon chain of the fatty acids seems to have a

greater affinity for similar protein groups, which may be responsible for the ability of these compounds to prevent, rather than to cause, the denaturation of serum albumin. This protein is characterized by a tendency to form stable complexes with a variety of compounds (44, 127, 127a-c), presumably by means of a favorable steric relationship of lysine and leucine residues (21, 44a), or other polar residues (127b).

The thymonucleate ion exerts a similar, though stronger, protecting action on egg albumin (31). Glycerol as well as sucrose and other non-reducing sugars and polyalcohols have been known for some time to combine with, and stabilize proteins in solutions (209, 6).

The adsorption of water vapor by proteins has recently been recognized as being at least partly dependent upon the chemical nature of certain protein groups. Pauling (176) on the basis of Bull's data (26) expressed the idea that the ability to adsorb water vapor was a function of the sum of all types of polar groups plus the proline residues of a protein. On the other hand, recent comparisons of various derived proteins has indicated that certain polar groups, particularly the amino groups, play a greater role in determining the affinity for water vapor than do others, e.g., carboxyl groups (171, 151).

Production of denaturation. The simplest and probably the most generally applicable and effective method is to dissolve the protein in 6-10 *M* urea, or preferably guanidine hydrochloride solution. Proteins differ greatly in their susceptibility to denaturing agents, particularly acid, alkali, alcohol, and heat. Thus no general conditions can be outlined which will cause extensive denaturation, but no other reactions in proteins. Recent careful studies of the heat denaturation of egg albumin (147) and β -lactoglobulin (24) may be helpful, but the denaturation of each protein is a separate problem.

Determination of isoelectric point of soluble proteins by means of detergents (182). To each of a series of 40 mg. samples of protein in 4 ml. 0.1 *N* solution of a buffer covering the expected isoelectric range is added 10 mg. sodium dodecyl sulfate. The protein is precipitated at all pH's below the isoelectric point, while clear solutions are obtained above this point.

III. REACTIONS INVOLVING PRIMARY BONDS

Protein reactions are studied either for preparative purposes, *i.e.*, to make protein derivatives, or in connection with protein group analysis. Many reagents are used for both purposes. In the present discussion, the preparative use of various agents will be

primarily considered, followed by a brief survey of the analytical methods that can be applied directly to proteins.

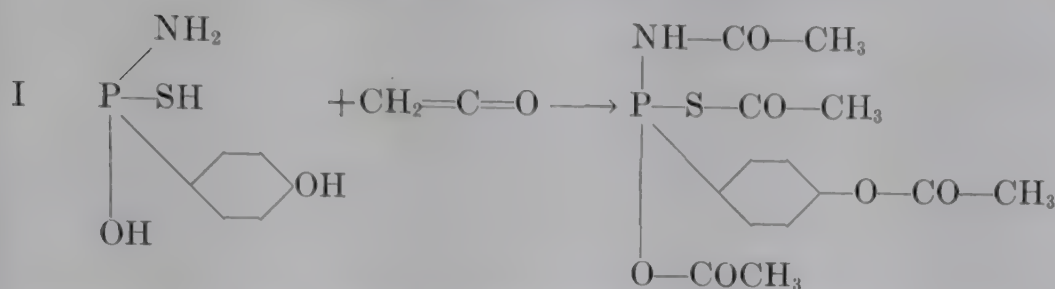
Chemical modification of proteins usually serves either of two ends. Biologically active proteins have been modified in a search for the structural localization of their activity or activities. Proteins have also been subjected to various reactions in the hope, sometimes gratified, of suppressing unwanted without interfering with the desirable qualities. For industrial proteins the latter has been the prime aim, with particular reference to physical properties, such as water resistance, strength, and plasticity. It is obvious that the different purposes have led to the development of two classes of derivatives, although the same reagents are often used. The modification of biologically active proteins has stimulated the search for specific reagents acting under mild conditions. On the other hand, the industrial aim has often called for reagents or conditions causing the modification of a maximal number of protein groups without regard to the integrity of the protein. Only a few examples of the preparation of exhaustively modified proteins will be given here, and the main emphasis will be placed on the use of presumably selective agents and biologically active proteins. However, no detailed survey of the extensive literature on this subject is intended. For this the reader is referred to two recent review articles (108, 172). In the present chapter, only those papers which contribute to our understanding of the mechanism of the reactions involved will be discussed.

A great number of protein derivatives have been prepared for immunological studies. Since the nature of the newly introduced determinant groups was generally found to be more important than the original protein or the mechanism of attachment, little work was done on the chemical aspect of these reactions. They will therefore be dealt with only briefly, the reader being referred to Landsteiner's book for a detailed discussion (129).

1. Acylation

a. Ketene

Acetyl derivatives have been prepared from a great number of proteins by means of ketene. When ketene gas was passed through an aqueous solution of pepsin at pH 4.0 to 5.5, it was found to react primarily with the amino groups, and at a slower rate with part of the phenolic and other, possibly the aliphatic hydroxyl groups (110, 107a) (see Formula I). Three acetyl derivatives were isolated



in crystalline form; one fully active and acetylated probably only on the amino groups (3 per mole); one about 60% active, with three of the 24 phenol plus indole groups acetylated; and one 10% active, with about 8-9 of these groups and 7-10 unknown groups acetylated, in addition always to the amino groups.

Insulin was found to react similarly, *i.e.*, its amino groups appeared to react much more readily than its phenolic groups, but the possible participation of other groups in the reaction was not studied (220b). As in the case of pepsin, insulin was not inactivated by acetylation of its amino groups, but lost its activity in the course of further ketenization. The conclusions from these two studies, since corroborated by independent evidence, are that the amino groups of pepsin and insulin are not, and the phenolic groups are,

TABLE II
ESSENTIALITY OF AMINO OR PHENOLIC GROUPS FOR
BIOLOGICAL ACTIVITIES^a

	Phenolic	Amino	Other Groups
Beta Amylase (barley)	probably, K, N (235)	no, K (235)	-SH (235)
Amylase (pancreas)		yes, K (140a)	
Pepsin	yes, K (110, 107a)	no, K (110, 107a)	
Adrenocorticotropic hormone	yes, I (107b, d)		
Gonadotropin (chorionic)	yes, I, K (135b)	yes, K (135b)	
Insulin	yes, K (135a)	no, K (135a)	
Lactogenic hormone	yes, I (94, 66c)	no, AA (66c)	-S-S- (228)
Diphtheria toxin	yes, K (220b)	no, K (220b)	
Tobacco Mosaic Virus Protein	yes, I (134)	yes, K (133)	
Trypsin	yes, K (174)	yes, K (174)	
Ovomucoid	no, ^a K, P, etc. (157a, b, 205)	no, ^a K, P, etc. (157a, b, 205)	SH not (3d)
Crotoxin	no, I, etc. (58b)	no, AA, etc. (58b)	-S-S- not
	yes, I (58b)	no, AA, etc. (58b)	-S-S-, -COOH
			-S-S- (216)
			-COOH, etc.

^a "Essentiality," as used in this connection is meant to indicate that modification of a considerable proportion of the respective groups destroys the specific biological activity. Only those studies in which analytical data on the nature and number of groups involved are available have been included in this table. Even in these cases it is often difficult to decide which groups are "essential": a. because of the lack of selectivity of ketene, the reagent used for most of these studies; and, b. because inactivation may begin to occur only after a certain fraction of several types of groups (some unknown) have been modified, as is the case for tobacco mosaic virus protein.

The reagents used have been abbreviated:

K = ketene N = nitrous acid I = iodine P = phenyl isocyanate AA = acetic anhydride.
The figures in parentheses give the literature reference.

essential for their ability to exert their particular biological activities (Table II).

These investigations have established the unfortunately erroneous belief that ketene treatment for a few minutes causes acetylation of all, and none but, the amino groups of proteins in general, while subsequent hours of reaction lead to acetylation of the phenolic groups exclusively. Recent studies of the ketenization of various proteins (195a) and of pituitary (133, 135b) and parathyroid hormone (241) have definitely established that the two types of groups may react at similar rates; surprisingly the phenolic groups of lactogenic hormone appeared to react more extensively in a brief ketenization at pH 6–7, than did the amino groups (75 and 35%, respectively) (133). Determinations of the total amount of acetyl bound, which alone can establish the non-participation of other groups, have not generally been performed. Miller and Stanley (157a) found that the small amount of acetyl bound by tobacco mosaic virus after exhaustive ketenization, corresponded approximately to the sum of the substituted amino and phenolic groups. In contrast, Sandor and coworkers (199–201) found serum proteins to bind much more ketene than was accounted for by the decrease in amino nitrogen; the phenolic groups did not react, thus other groups must have been involved.

Ketene is known to react with thiols (163b) and has been shown to acetylate the available —SH groups of egg albumin (57, 45a) (Formula I). A slow acetylation of the aliphatic hydroxyl groups has recently been demonstrated.⁵ On the other hand, there is no evidence for the action of ketene on amide, guanidyl, or imidazole groups. The specificity of this and other reagents are summarized in Table III.

Of the readily acetylated groups, the amino groups alone give comparatively stable acetyl compounds, while phenol and thiol

⁵ On the basis of model experiments with glucosamine (13), a carbohydrate (163a), and hydroxyglutamic acid (163b), it is generally assumed that hydroxyl groups do not react with ketene. On the other hand, this possibility was definitely indicated by the earlier literature (217). Treatment of silk fibroin with ketene gas was found to introduce acetyl groups corresponding in number to the sum of the phenolic and aliphatic hydroxyl groups (126). Recent experiments of Olcott and Fraenkel-Conrat also have demonstrated a slow acetylation of the aliphatic hydroxyl groups of proteins and model substances in aqueous solution at room temperature (59, 172). On the other hand, the small amounts of acetyl bound by gliadin and protamine is evidence that the amide and guanidyl groups did not react under the rather severe reaction conditions chosen for these experiments (172). Herriott (107a) found ketene to decrease the Folin chromogenic value of tryptophan and glycyl tryptophan in a manner not reversible by exposure to alkali. This is probably due to acetylation of the free amino group and no evidence for acetylation of the indole ring. Thus, we have found α -N-acetyl tryptophan to have a lower molar chromogenic value than tryptophan.

TABLE III (from 172)

EFFECTS OF REAGENTS ON PROTEIN GROUPS UNDER
CONDITIONS MOST FAVORABLE FOR SPECIFICITY*

	Amino	Guanidyl	Imidazole	Indole	Aliphatic Hydroxyl	Amide	Thioether	Disulfide	Sulfhydryl	Phenol	Carboxyl
Oxidizing Agents											
Iodosobenzoate, porphyrindin	—	—	—	—	—	—	—	—	3 +	—	—
ferricyanide, iodine ^a	—	—	—	—	—	—	—	—	—	—	—
Hydrogen peroxide ^b	—	—	—	—	—	—	2 +	—	3 +	+	—
Tyrosinase ^c	—	—	—	—	—	—	—	—	—	—	—
Reducing Agents											
Cysteine, thioglycollic acid, thioglycol, etc. ^d	—	—	—	—	—	—	—	3 +	—	—	—
Cyanide, sulfite	—	—	—	—	—	—	—	3 +	—	—	—
Alkylating Agents											
Iodoacetate, iodoacetamide ^e	—	—	—	—	—	—	—	—	2 +	—	—
Dinitrofluorobenzene ^f	3 +	—	+	—	—	—	—	—	+	3 +	—
Acylation Agents											
Ketene ^g	3 +	—	—	±	±	—	—	—	+	2 +	—
Acetic anhydride ^h	3 +	—	—	—	—	—	—	—	—	±	—
Phenyl isocyanate ⁱ	3 +	±	—	—	—	—	—	—	3 +	?	—
Carbon suboxide ^j	2 +	—	—	—	—	—	—	—	3 +	2 +	—
Azides, benzoyl-, carbobenzoxy- benzenesulfonyl chlorides, etc. ^k	3 +	—	—	—	—	—	—	—	+	±	—
Concentrated sulfuric acid ^k	—	—	—	—	3 +	—	—	—	3 +	+	—
Nitrous acid ^l	3 +	—	—	+	—	—	—	—	3 +	2 +	—
Iodine ^m	—	—	±	+	—	—	—	—	3 +	3 +	—
Formaldehyde (pH 7-8) ⁿ	(3 +) ⁿ	2 +	—	+	—	—	—	—	±	—	—
Formaldehyde (pH 11) ⁿ	(3 +) ⁿ	2 +	—	3 +	—	3 +	—	—	+	—	—
Epoxydes ^o	+	—	—	—	—	—	—	—	2 +	2 +	3 +
Mustard gas ^p	±	—	+	—	—	—	+	—	3 +	?	2 +
Acid-alcohol ^q	—	—	—	—	—	—	—	—	—	—	3 +
p-Chloromercuribenzoate, etc. ^r	—	—	—	—	—	—	—	—	3 +	—	—
Diazonium compounds ^s	—	—	3 +	+	—	—	—	—	—	2 +	—

^a pH 7, 0.001–0.01 *M*, 0–25°, 5–30 min. The specificity of iodine as an oxidizing agent requires a high concentration of iodide ions, pH 1–7.

^b pH 6.6, 0.005 *M*, 25°, 0.5–40 hours.

^c pH 7.3, 24 hours.

^d pH 7–8, 0.001–0.1 *M*, 25°, 0.5–4 hours.

^e pH 7–8, 0.05–0.1 *M*, 25°, 0.5–2 hours.

^f pH 7–8, 0.17 *M*, 25°, 2 hours.

^g pH 5–8, 0–25°, 5–30 min.

^h pH 7–8, 0°, 30 min.

ⁱ pH 7–8, 0.25°, 0.5–2 hours, reagent-protein ratio, 0.5–2.5:1.

^j pH 7–9, 0–25°, 0.5–2 hours, limited amounts of reagent.

^k –13°–0°, 10–30 min.

^l pH 4, 0°, 30 min., 1 *M* nitrite.

^m pH 5–11, –5–25°, 0.5–3 hours, limited amount of iodine, low iodide concentration. See also footnote a.

ⁿ 25°, 1–2 *M*, at pH 7–8, 1 hour; at pH 11, 10 min. Amino groups react rapidly but reversibly. After isolation by dialysis, amino groups are essentially free.

^o pH 5–6, 1–2 *M*, 25°, 1–4 days.

^p pH 5–6, 25°, 0.5–4 hours.

^q 0.01–0.1 *M* mineral acid in absolute alcohol, 0–25°, 1–2 days.

^r pH 7, 10^{–5} to 10^{–3} *M*, 25°, 5–30 min.

^s pH 7–9, limited amounts of reagent, 25°, 30 min.

* The symbols used have the following significance: 3 +, 2 + and + indicate the relative rapidity or extent of reaction with 3 + denoting the most rapid reaction. ± indicates that reaction may or may not occur under the conditions suggested. — indicates those reactions either that have been shown not to occur or appear improbable from organic chemical considerations. ? indicates those reactions for which more information is required. Spaces have been left blank where there is a possibility of reaction but no evidence is available. For references to the original literature, see Table II and the text. Allocation of the proper symbols for each reaction is difficult because of the differences between proteins. The choices are arbitrary.

acetates are quite labile in acid and even more so in weakly alkaline solution at room temperature. Aliphatic acetates are hydrolyzed in slightly more alkaline solution. This lability of the ester-bound acetyl groups makes it possible to prepare purely amino-acetylated proteins even in cases where the phenolic and amino groups react at similar rates, by exposing the ketenized product to either acid

or alkali. It appears that this is the favored method even in the case of pepsin (107a). However, in many proteins far from all amino groups react with ketene under the usual experimental conditions. A further drawback of this reagent is its tendency to denature those proteins which are sensitive to surface forces, *e.g.*, egg, and to a lesser extent, serum albumin.

b. Acid anhydrides, chlorides, and azides

Treatment with acetic anhydride in aqueous solution of pH 7–8 at low temperature appears to represent both a selective and effective means of acetylating most of the amino and hardly any phenolic groups in the proteins studied (serum and egg albumin, insulin, lysozyme, trypsin and others) (172, 58b); the masked —SH groups of egg albumin also remain unaffected. This technique appears to cause no denaturation, since the acetyl derivative of egg albumin is isoelectrically soluble and heat coagulable. It must be stressed, however, that the observation that acetic anhydride represents a specific reagent for protein amino groups is limited to the conditions and to the proteins studied. Upon application of this method to other proteins, analyses of the groups involved are definitely indicated.

Benzoic anhydride and *m*-chlorobenzoyl chloride were used by Goldschmidt and coworkers (79, 80) as protein reagents in weakly alkaline solution. They introduced up to 12 equivalents of the reagent into egg albumin and showed that part of the acyl residues were attached to the ϵ -amino groups of lysine, and quite resistant to hydrolysis, while most were to varying degree labile in alkali at low temperatures. Abderhalden and Schmitz (2) made a similar study of the stability of fixation of benzoyl residues. Felix and coworkers (52, 46) demonstrated that benzoyl chloride and *m*-chlorobenzoyl chloride could react quantitatively with the guanidyl and imidazole groups of protamines under the conditions of the Schotten-Baumann technique. Nevertheless, benzoyl chloride has recently been used to good advantage for the almost selective acylation of 72% of the amino groups of casein (151). This was achieved in weakly alkaline solution by careful addition of the equivalent amount of the reagent.

Carbobenzoxychloride has been used in similar manner for the acylation of insulin (74b) and tobacco mosaic virus protein (157b). This reagent acylated the same number of amino but fewer phenolic groups than did ketene, phenyl isocyanate, *p*-chlorobenzoyl chloride, or benzene sulfonyl chloride (157b).

Acetic and higher fatty acid anhydrides and chlorides have often been used under anhydrous conditions, with or without the addition of pyridine, and at temperatures ranging from 0° to 125° (1, 99b, 105, 29, 82, 15, 25, 83). Under the more rigorous conditions, aliphatic and phenolic hydroxyl groups participate in the reaction and probably also most of the basic groups, leading to derivatives containing 15–34 equivalents of acetyl (up to 15%) or other acyl residues. Benzoic and phthalic anhydride have recently been employed under anhydrous conditions in pyridine solution at elevated temperature to introduce a maximal number of acyl residues into various proteins (171). These, as well as the aliphatic fatty acid derivatives (83, 83a), may find industrial applications.

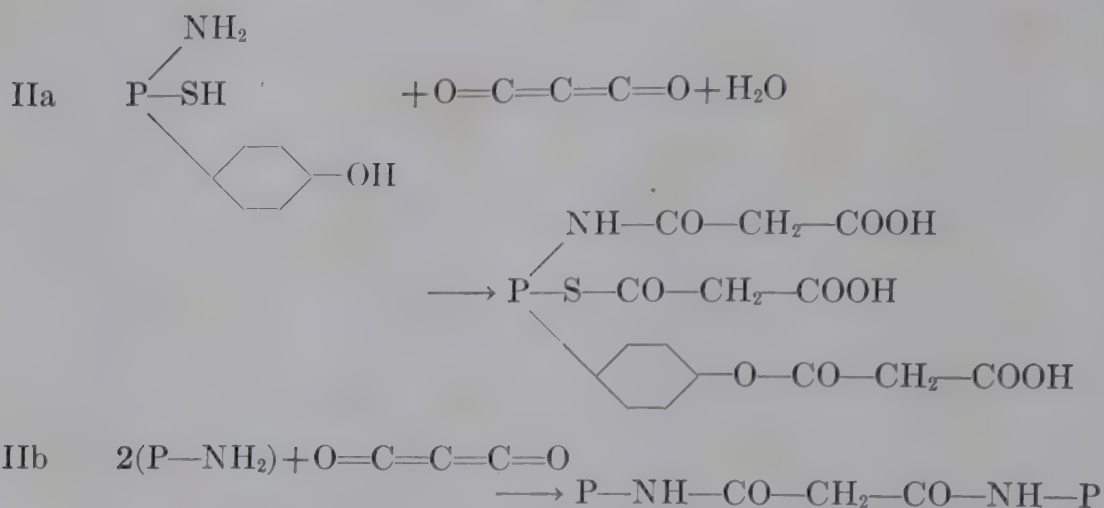
Acid azides have been used successfully to introduce various complex acids and amino acids into proteins. Their advantage over acid chlorides and anhydrides lies in the ease of preparation (esters → hydrazides → azides), and in that no strong acid accumulates during the coupling reaction (38, 39, 116, 153). Substituents containing aromatic rings may also be introduced into proteins through oxazolone derivatives (130).

Technique of selective acetylation of amino groups

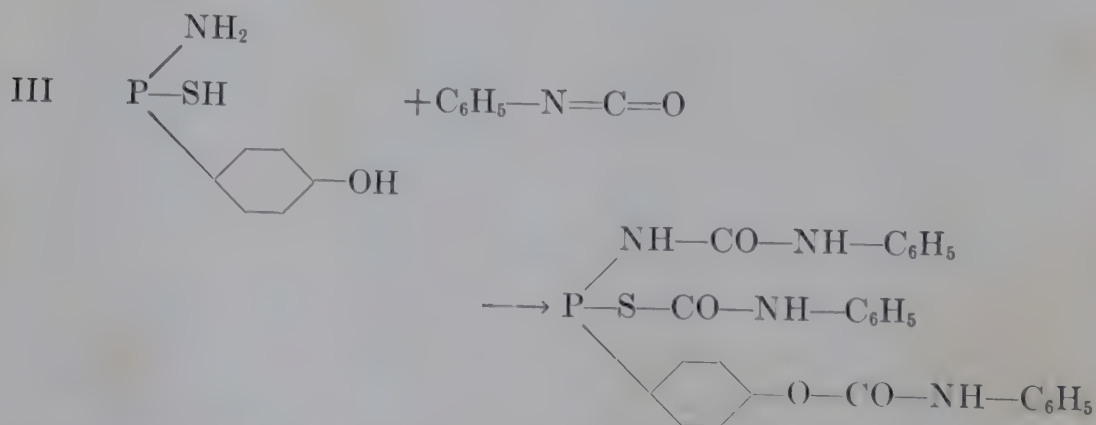
To 1 g. of protein, dissolved or suspended in 20 ml. of half to fully saturated sodium acetate solution and cooled in an ice bath, is added with shaking or stirring in several portions and over about one hour a total of 1.2 ml. of acetic anhydride. After the odor of the reagent has disappeared, the protein derivative is isolated without loss by dialysis. The amino nitrogen ranges from 5–40% of the original for various proteins. The Folin color value is 80–90% of that of the original protein, and 90–100% of that obtained after exposing the protein for 10 minutes at pH 11 (107a, cf. also p. 577). Analyses for acetyl introduced indicates amounts equivalent to losses in amino nitrogen.

c. Carbon suboxide and aromatic isocyanates

These reagents, like ketene, have cumulated double bonds. They also resemble ketene in their effects on proteins. Carbon suboxide reacts at pH 7.5 at approximately similar rates with the amino and phenolic groups of the proteins studied (224a) (Formula IIa). At pH 5–6 the —SH groups of egg albumin react faster than either of these groups (58). The malonyl half ester bonds formed with the phenolic groups are more labile than the corresponding acetyl esters but the S-malonyl groups hydrolyze somewhat more slowly (194, 224a, b, 58). Model experiments with amino acids suggest that guanidyl, imidazole, and aliphatic hydroxyl groups do not react with carbon suboxide (195).



Phenyl isocyanate has often been used in the belief that it reacted selectively with the amino groups in aqueous solution buffered around pH 8 and at temperatures ranging from 0° to 25°. This belief was based on the work of Wormall and coworkers (114a, b, c, 74a) who found that treatment of serum globulins, casein, gelatin, and insulin with *p*-bromophenyl isocyanate introduced amounts of bromine into these proteins which were approximately equivalent to their not too reliable amino nitrogen analyses. More recent studies on tobacco mosaic virus protein (157a), egg albumin (57) and urease (45a) have demonstrated the participation of sulfhydryl groups, in the reaction with phenyl isocyanate under similar conditions⁶ (Formula III).



Isocyanates have been used for the introduction of various aromatic and heterocyclic groups into proteins. Two 1,2-benzanthryl

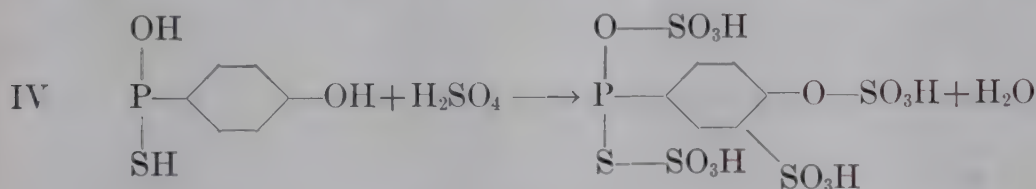
⁶ The involvement of guanidyl (74a, 92) and reactive amide groups (119, 62) in the reaction has also been suggested. Recent experiments (172) have shown that the —SH groups of egg albumin react even at 0, at pH 7.5 more readily than at pH 4.5, and at both pH's more readily than the amino or phenolic groups, with *m*-chlorophenyl isocyanate. The participation of the phenolic groups (157a) is subject to some doubt in the light of recent evidence for the unreliability of Folin color values obtained on intact protein derivatives (109, 60).

isocyanates seemed to react exclusively with part of the amino groups of horse serum albumin when added in dioxane solution at pH 8.2 and at 0 to 10° (42). Rodney and Fell (192) introduced histamine residues into blood proteins in similar manner.

Under anhydrous conditions at elevated temperature, and preferably with pyridine as catalyst and solvent, phenyl isocyanate reacts with all types of protein groups carrying active hydrogen atoms (not with the peptide bonds). The resulting products contain up to 30% of the reagent and have a very low affinity for water, in contrast to the untreated proteins (61).

2. Sulfation and Phosphorylation

Treatment of proteins with concentrated sulfuric acid at low temperatures causes the almost instantaneous and quantitative transformation of the aliphatic hydroxyl into acid sulfate ester groups (Formula IV). The only other groups participating in the



reaction are the —SH and part of the phenolic groups. If the reaction is performed entirely at -18° , only hydrolyzable phenolic sulfate esters are formed, but if the reaction mixture is permitted to come to or to stand at room temperature, progressively more ring-sulfonation occurs (187) (Formula IV). Part of the guanidyl groups appear to be destroyed if the reaction is allowed to proceed for 24 hours (146), but not under the conditions used by Reitz, *et al.* (187).

The sulfation reaction leads to very acid protein derivatives, some of which may prove to have useful physical properties (188). The finding that insulin completely sulfated in this manner retains its hormonal activity appears noteworthy and may render this technique less repulsive to the biochemist (76).

Another method of sulfation, by means of the addition product of pyridine and chlorosulfonic acid at elevated temperature introduces considerably more sulfate residues into proteins than does concentrated sulfuric acid. Amide, amino, guanidyl, phenolic, indole, sulfhydryl, and aliphatic hydroxyl groups were found to be sulfated (or sulfamated) under these conditions (189).

Phosphorylated proteins have been prepared by careful treatment with phosphorus oxychloride at or above pH 8.5. The reaction affects part of the amino and probably of the phenolic groups. An

appreciable fraction of the phosphate is bound in a more labile manner than it is in natural phosphoproteins, *e.g.*, casein; another fraction is bound more firmly. Thus, only a fraction of the phosphate residues appear to be bound by the serine hydroxyl groups (101, 149). Indications have recently been obtained that treatment of proteins with phosphorus pentoxide in 100% phosphoric acid at room temperature for several days may lead to phosphorylation of the aliphatic hydroxyl groups of proteins (54a).

The remarkable ability of diisopropyl fluorophosphate to inhibit esterases has been intensively studied (147b). Chymotrypsin loses its activities (both protease and esterase) when one mole of the reagent is bound with the liberation of hydrogen fluoride (118a). The site of the reaction on the protein remains to be elucidated.

3. Alkylation, Arylation, Esterification

Diazomethane, methyl sulfate, and methyl halides have been used by many investigators for the modification of proteins. The more recent work has mainly been concerned with the methylation of wool, silk fibroin, gelatin, and insulin (14, 15, 17, 82, 148, 196). While some of the products may represent useful modifications (196), the reagents in general appear quite non-specific in their action, causing methylation of sulfhydryl, amino, phenolic, carboxyl, and possibly other groups. Methyl bromide or sulfate treatment of various proteins at pH 8 and room temperature introduced amounts of methoxyl into three proteins corresponding approximately to their carboxylic acid groups (14, 17, 17a). In contrast, studies on insulin suggest that diazomethane, but not methyl iodide, tended to methylate carboxyl groups prior to combining with other types of groups (69, 34). Methyl sulfate is believed to methylate some peptide bonds, activated by adjacent hydroxyamino acid residues, with concomitant introduction into the protein of the sulfate moiety as sulfate ester of the hydroxyl group (14, 17). In strongly alkaline solution this reagent may cause the transformation of all phenolic (36) and part of the serine hydroxyl groups of proteins into methyl ether groups (82).

Treatment of proteins with methyl alcohol containing small amounts of mineral acid leads to selective esterification of the carboxyl groups (64). In many proteins the carboxyl groups have been quantitatively methylated without any detectable participation of other groups in the reaction. Higher alcohols react progressively less readily.

This method of esterification was first introduced by Carr and co-workers (30) but was later discredited as a specific reaction owing to analytical difficulties (33), since clarified (76). Felix and co-workers (54, 46) have used strong alcoholic hydrochloric acid to esterify protamines, histones, and gliadin. Typical proteins probably cannot withstand this treatment without damage and breakdown (124, 64).

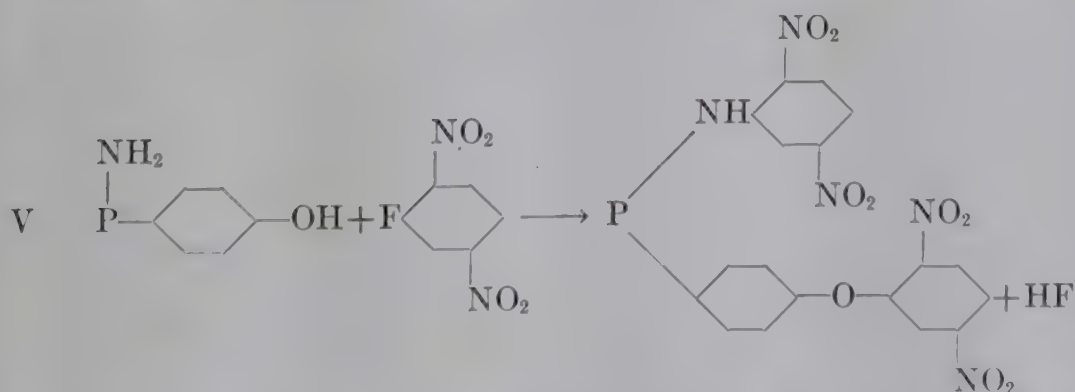
Technique of selective and extensive methylation of carboxyl groups (64)

One hundred mg. protein is suspended in 10 ml. methyl alcohol to which is added 0.1 ml. concentrated aqueous hydrochloric acid. Many proteins dissolve within a few seconds; they may later set to a gel or precipitate. After several days' standing at room temperature, the protein derivative is isolated either by precipitation and washing with ether, or by evaporation of the methanol and HCl at room temperature, or by dilution with water and dialysis. Lyophilization of an aqueous solution or suspension is an essential step if methoxyl analyses are to be performed.

The entire reaction may be performed at 0° or lower temperatures. Egg albumin was extensively methylated but also denatured at -5°; serum albumin was half esterified and apparently not denatured (172).

With lower acid concentration, e.g., 0.01 *N*, methylation is less complete, but the resulting protein esters are generally soluble in methanol containing such small amounts of hydrochloric acid.

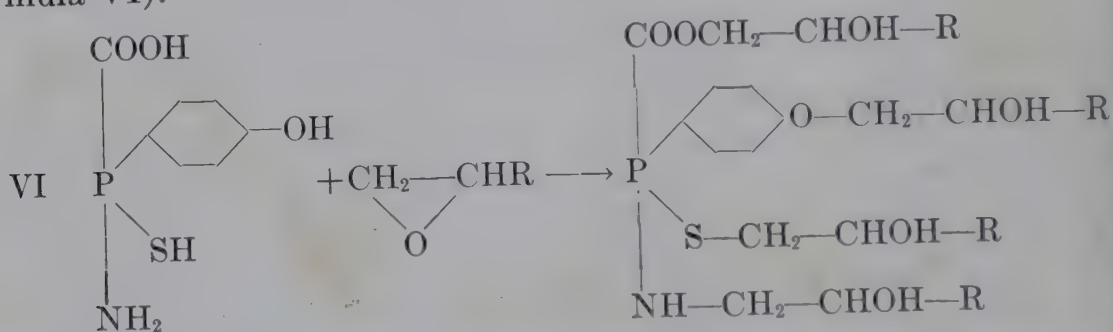
1,4-Dinitrofluorobenzene has recently been shown to combine readily with proteins in bicarbonate solution (202a) (Formula V).



The amino groups react almost quantitatively, but in addition the phenolic, sulfhydryl, and imidazole groups of most proteins (180a) may also become substituted. The great advantage of this derivative is that the resulting bonds are comparatively resistant to acid hydrolysis. Furthermore, the *N*-dinitrobenzene derivatives are

colored and can be separated chromatographically from one another and from other products of hydrolysis. In this manner, Sanger was able to identify the residues bearing free amino groups (both the ϵ -group of lysine and the terminal α -amino group) in insulin (202a) and gramicidin S (202b).⁷

1,2-Epoxydes have been found to esterify carboxyl groups of proteins under very gentle conditions, *i.e.*, at room temperature in neutral aqueous solution (58). Their action, however, appears not to be specific. The phenolic and sulfhydryl groups become etherified. The amino groups are alkylated if the reaction is performed in slightly alkaline solution, but only to a small extent at pH 3.5. Since the reaction abolishes the polarity of the acid but not that of the basic groups, the physicochemical properties of the epoxide derivatives resemble those of selectively esterified proteins (Formula VI).

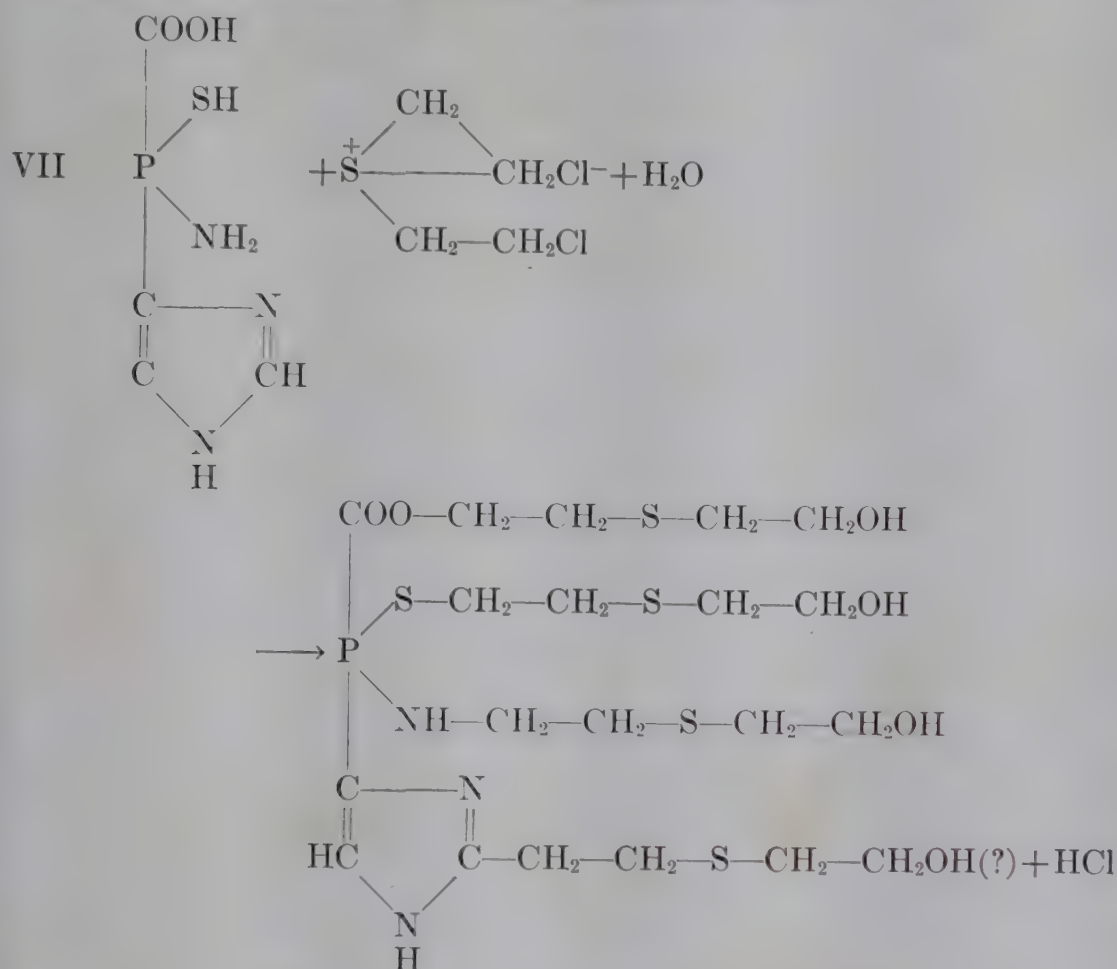


The chemical interactions of some war gases with proteins have been extensively studied in recent years. In general, it appears that mustard gas and the nitrogen mustards act in aqueous media not as chloroalkyl compounds, but in the form of sulfonium or imonium ions (81, 109, 217c). These, in the case of the nitrogen mustards, are formulated as three-membered rings (Formula VII). They resemble epoxides in their reactivities toward the various active hydrogen carrying groups. In solution of pH 5.5–6.0 esterification of the carboxyl groups appears as the predominant reaction (109, 160c), although the esters of the nitrogen mustards may be very unstable (73). In weakly alkaline solution, amino and imidazole groups become alkylated (73, 97a, 125, 160a, 221a). Other protein groups which may react are the available sulfhydryl (9, 97a, 108, 178a) and the methionine thioether groups; the latter reaction is suggested by model experiments with the amino acid and a peptide (217a, b).⁸

⁷ Benzene sulfonylchloride (91), toluene sulfonylchloride (36), and phenyl isocyanate (119) have been used for the same purpose, although not quantitatively.

⁸ Evidence for the involvement of the phenolic groups was also obtained (109). But the agreement between the amount of reagent introduced and the number of carboxyl groups esterified, as determined by titration, led Herriott, Anson, and

For a further discussion of the effects of alkylating agents on proteins, see the section on —SH reagents (p. 561).



4. Deamination

Nitrous acid has long been recognized as one of the most useful reagents available to the protein chemist. It is primarily an analytical tool, because of the ease with which it transforms amino into hydroxyl groups with liberation of one mole of nitrogen. The quantitative aspects of this reaction will be discussed under analytical methods.

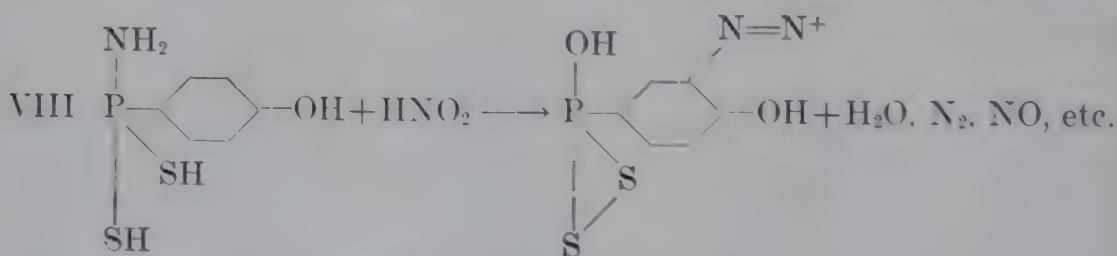
As a preparative reagent, nitrous acid is of limited value because of its lack of specificity. The phenolic and imidazole groups have

Northrop to believe that the decrease in Folin color was an artefact rather than proof for the etherification of the phenolic groups.

The same possibility has been recognized in the study of other derivatives (155, 59). Thus, in general, a decrease in the Folin chromogenic activity of an intact protein should not be accepted as absolute proof for the participation of its phenolic and/or tryptophan residues in a reaction. It may represent but an indication of their unavailability to the reagent. The question whether the groups are actually modified can in many cases be ascertained by performing the analyses after enzymatic breakdown of the protein. Many phenolic derivatives, such as etherified or iodinated ones, are resistant even to acid or alkaline hydrolysis. On the other hand, the acetyl and malonyl phenolic linkage cannot even withstand the conditions of enzymatic digestion at pH 5 or 8. This renders accurate analyses of the extent of such acylation of phenolic groups impossible.

definitely been shown to participate in the reaction (131, 221, 237, 179a). A slow reaction of the guanidyl groups has repeatedly been demonstrated (221, 197, 137, 47). The —SH groups are reversibly oxidized by nitrous acid under gentle conditions (235). Furthermore, model experiments with cystine and other disulfides (141), as well as with skatole (55) and tryptophan (203, 49, 179a) have suggested that these amino acid residues may also be affected during the prolonged action of nitrous acid on proteins. A decrease in the cystine content, as determined by the Sullivan reaction, was observed after the deamination of wool (111a). In contrast to Philpot's finding with pepsin (179a), experiments with insulin and bovine serum albumin have shown that even treatment with nitrous acid under the mildest conditions (0° , pH 4, $\frac{1}{2}$ to one hour) suffices to affect some of the phenolic groups while not abolishing more than half of the amino groups (172). Thus, no conditions can at present be proposed which will insure a selective deaminating action of this reagent on any protein with the possible exception of pepsin (179a) and gelatin (113).

Of the secondary actions of nitrous acid on proteins, only that on the phenolic groups has been studied in some detail (161, 179a, 140b). A nitroso derivative is the intermediate, and a derivative with a diazo group in the ortho position to the hydroxyl the more stable product (Formula VIII). The latter couples with added



phenols and probably also with reactive protein groups (see p. 559), which may account for the red brown color of all "deaminated" proteins.

According to Philpot and Small (179a), the reactions involving the phenolic groups proceed at a slow rate and are of the first order in the presence of excess nitrite, in contrast to the rapid, second order reaction of the amino groups. Thus, studies of the rate and the kinetics of inactivation of biologically active proteins with nitrous acid have often been interpreted as evidence for the essentiality of the amino or phenolic groups for the activity of the protein (214a, 140a, 235) —at times without due regard to the possible role of the indole, imidazole, and other residues. It must also be stressed

again that the reactivity of the same group may vary greatly from one protein to another, and even more so for the amino acid residue in proteins as compared to the free amino acid. Thus, no conclusions should be based on reaction rates alone. For each protein, differences in the reactivity of the various groups should be presupposed, and all studies involving changes in these groups should be supported by analytical data.

5. Iodination

Acylating agents and nitrous acid have been shown to act more readily on the amino groups than on the phenolic groups. Iodine may serve the complimentary purpose of affecting the phenolic but not the amino groups. In neutral or alkaline solution, iodine readily transforms all available tyrosine residues into 3,5-diiodotyrosine. Careful analytical work has demonstrated that its substituting action is confined to these groups in the case of zein (162), insulin (94), pepsin (107b, d), lactogenic hormone (134), and serum albumin (132).⁹ However, this apparent specificity of iodination does not apply in the case of hemoglobin (12a, b), lysozyme and other proteins (58a), the imidazole groups of which participate even in neutral and acid solution. That the indole groups may become oxidized by iodine at pH 8 has recently been indicated (191b, c). The proteins with which iodination had previously been studied in detail contained either no tryptophan (zein, insulin) or very little in comparison to the tyrosine present. Chymotrypsinogen, a protein containing more tryptophan than tyrosine, was found to bind no iodine at pH 3.5, in contrast to free tryptophan (3b). Unfortunately, this reaction was not studied in neutral or slightly alkaline solution.

In acid solution, iodine acts as an oxidizing agent on cysteine and, more slowly, on cystine, tyrosine, tryptophan and possibly other residues. These reactions will be discussed in connection with the action of other oxidizing agents. Under carefully controlled conditions, particularly at low iodide concentration, this oxidizing action may be largely suppressed, but this has not often been taken into due account. Fibrinogen was found to undergo oxidation at an appreciable rate even at 0° and at neutrality (128c).

⁹ Methods based on the Millon reaction, such as that of Lugg, may be used, but the Folin reagent is not suitable for the problem, because diiodotyrosine retains 50% of the original chromogenic activity of tyrosine by this technique (107d). Monoiodotyrosine, which may be formed in the initial stages of the reaction, gives strongly positive Folin and Millon tests.

A remarkable effect of iodination of proteins has recently been recognized, *i.e.*, the transformation of tyrosine into thyroxine residues (142). This proceeds upon treatment with iodine in weakly alkaline solution, the iodine or more probably the hypoiodite ion playing the dual role of substituent and oxidant. The mechanism of this interesting and practically important reaction has been studied in some detail in recent years (121, 95, 185).

Technique of complete iodination of insulin (94)

200 mg. crystalline insulin dissolved in 2 ml. aqueous ammonia (sp. gr. 0.880) and cooled in a freezing mixture, were treated with 0.2 ml. 2.8 *N* iodine in potassium iodide, added drop-wise. The resulting gel dissolved upon addition of water, was neutralized with 2 ml. glacial acetic acid and purified by reprecipitation at pH 4.5. Yield 90% of theoretical. Analysis showed 15.4% I, in agreement with the calculated value for diiodination of all the tyrosine, and 11.7% N.

Technique of iodination of available phenolic groups of native serum albumin (132)

A solution containing 12.5 mg. of protein per ml., 0.18 *M* pH 5.7 acetate, and 0.034 *M* potassium iodide was treated for 60 hours at room temperature with iodine (initial concentration 0.00516 *M*). The protein bound only 2.1 equivalents of iodine, equivalent to diiodination of 40% of the tyrosine residues. Colorimetric analysis indicated that 60% of the tyrosine had not reacted.

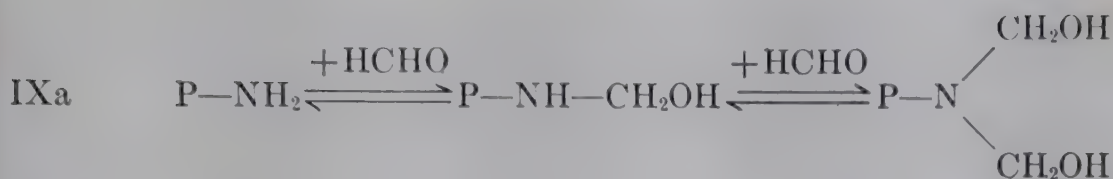
6. Reaction with Aldehydes

a. Formaldehyde

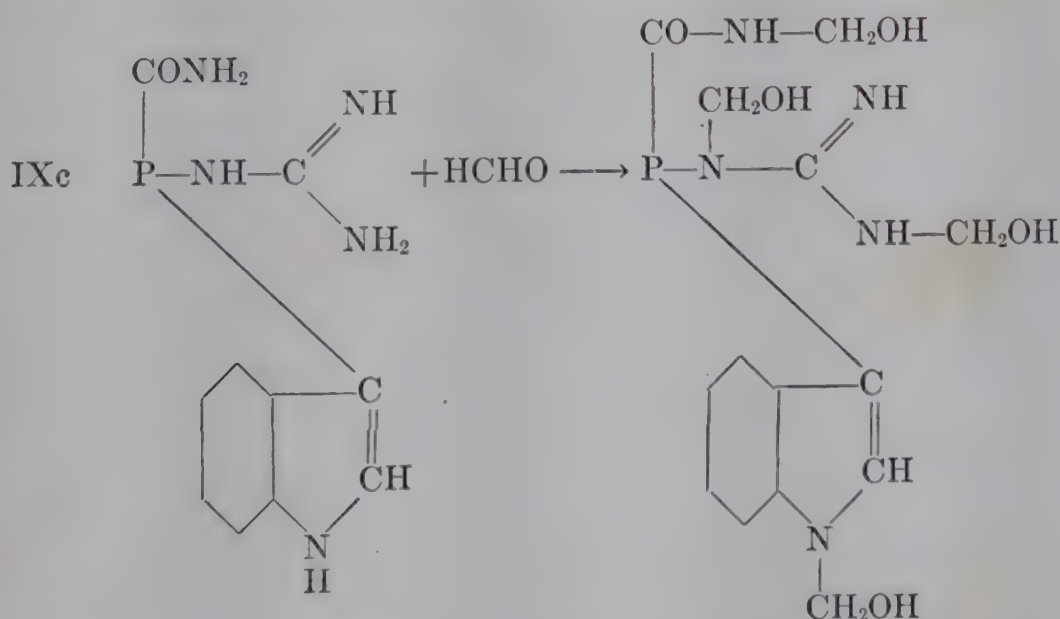
Formaldehyde is one of the oldest protein reagents, and possibly the only one that has found wide and manifold practical applications, ranging from the tanning of leather and the fixing of tissue to the preparation of diphtheria toxoid and influenza vaccines. Yet our understanding of the reaction was quite incomplete up to a few years ago, and its formulation is erroneous in most older textbooks and publications.¹⁰ Briefly, the reagent may be characterized as highly non-specific.

The most rapid reaction occurring at neutrality or in alkaline solution is an addition to the amino groups, leading to amino-methylol groups. Depending upon formaldehyde concentration, more or less of the dimethylol compounds will be formed (Formula

¹⁰ There is now available a lucid review of the action of formaldehyde on amino acids and proteins which corrects the old errors and discusses critically all evidence that was published at that time (68). The reader is referred to this review for a thorough presentation of the extensive literature on the protein-formaldehyde reaction.



IXa). But these reactions are readily reversible. Thus, the amino nitrogen content of proteins briefly treated with formaldehyde appears unchanged, and almost all the formaldehyde precipitates with dimedon at pH 4.6 and is released upon dialysis. Upon longer standing of the reaction mixtures, the methylol groups undergo further reactions leading to stable fixation of increasing amounts of the formaldehyde on the amino groups in a manner not reversible under the conditions of the dimedon test (231), amino nitrogen determinations, nor by dialysis (231, 168, 62, 174). Yet most of the formaldehyde thus introduced at room temperature and neutrality can be recovered upon acid hydrolysis of the proteins. The mechanism of this secondary semi-stable fixation of formaldehyde by the amino groups has only recently been elucidated (65b, 64b, 172) (Formula IXb, c).



In alkaline solution, three additional types of protein groups combine readily and, in contrast to the amino groups, stably with formaldehyde, namely, the guanidyl (112, 65a) and amide groups (62, 65b, 172), and the indole rings (59) (Formula IXc). Acid effectively catalyzes the reaction of formaldehyde with the amide (243, 62) and to a lesser extent of the guanidyl and indole groups

(65b, 172). But at elevated temperatures all these groups probably react appreciably at neutrality, the guanidyl groups binding up to two equivalents of formaldehyde (65a).~

This probably completes the list of protein groups which bind formaldehyde readily, with the exception of the —SH groups which may react if they are available (3c); most of those of native egg albumin do not react at room temperature and neutrality (172).

At temperatures of 70° or higher, the effects of formaldehyde become yet more complex. The disulfide groups are in part reduced by the aldehyde and then combine with it, some yielding —S—CH₂—S— linkages as they occur in djenkolic acid (154a, 40). That the reactive disulfide bonds of keratins also participate in the formaldehyde reaction in alkaline solution (100) is not surprising.

Besides primary reacting groups there are others which do not react directly with formaldehyde under mild conditions, but are able to condense with the resulting methylol groups. Techniques to study these secondary reactions have only recently become available (169, 59). If the imino-methylol group condenses with reactive —CH groups on the imidazole, phenolic, and indole residues, stable carbon-links are introduced, a reaction type which may account for part of the irreversible binding of formaldehyde by proteins (169, 64b) (Formula IXb). (The methylol groups introduced directly into indole residues in alkaline solution are also quite resistant to acid hydrolysis.) Another important condensation reaction involves pairs of amino and amide groups and probably accounts for the slow disappearance of amino groups in a manner which is not reversed by dialysis or during the amino nitrogen analysis (62, 65b, 172) (Formula IXc). These secondary cross-linking reactions appear to proceed slowly over a wide pH range (pH 2–8). They are probably responsible for the tanning and hardening effects of formaldehyde and may also play a role in vaccine formation. In addition, the combination of the reagent as methylol with indole, guanidyl, or amide groups may effect changes in the biological properties of proteins (e.g., 59, 131a).

The recently discovered crosslinking action of formaldehyde between amino and amide (or guanidyl) groups has supplied a means of modifying specifically either the amino or the amide+guanidyl groups of proteins. This is achieved by treating the protein with formaldehyde in the presence of a great excess (e.g., a five-fold, by weight) of a simple amine or amide, at pH 4 to 7. Thus, in the presence of alanine, the protein amide and guanidyl groups become largely substituted by —CH₂—NH—CH(CH₃)—COOH groups,

while the protein amino groups are, by mass law action, prevented from entering into crosslinking reactions and are free after dialysis. In contrast, added acetamide becomes attached, through methylene, to the amino groups and protects the protein amide and guanidyl groups from participating in the reactions (65b).

This brief summary of our present understanding of the protein-formaldehyde reaction may well be concluded with a quotation from the review of the subject by French and Edsall (68): "The remarkable variety of these reactions, indeed, has not always been fully realized by those who have employed formaldehyde as a reagent. Some of the reactions are rapid, some are slow; some are readily reversible, some practically irreversible; some proceed readily even at room temperature, others only at higher temperatures."

b. Other aldehydes

Acetaldehyde and other aliphatic aldehydes as well as benzaldehyde have also been used for protein modification (193a, 69). Acetaldehyde caused extensive "browning" (see below); it reacted primarily with the amino groups (160b). Reaction with the indole groups in alkaline solution has recently been demonstrated with gramicidin (63).

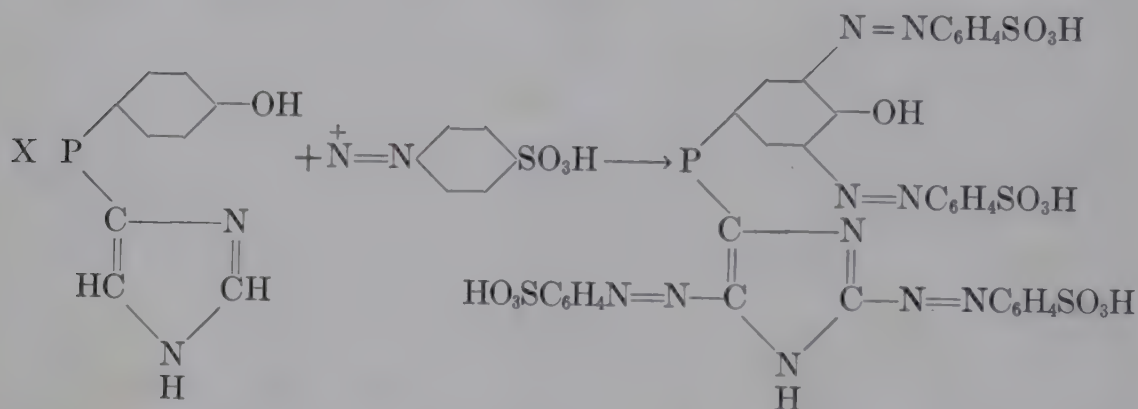
The reaction between proteins or protein breakdown products and aldoses, primarily glucose, appears to play an important role in the deterioration of many types of dehydrated and preserved foods. This so-called "browning reaction" is at present receiving much attention. In the case of proteins, amino groups are certainly the foci of primary attachment of the aldose (181, 170, 11, 106, 129a, 160a), but the mechanism of the browning is not yet understood.

7. Diazotization and Coupling

The —CH groups in ortho position to the phenolic groups of tyrosine and to the imino groups of tryptophan and histidine have in common a great reactivity toward certain reagents. As previously mentioned, these groups tend 1. to condense with methylol groups (formed by addition of formaldehyde to amino and similar groups) to yield $\text{—CH—CH}_2\text{—NH—}$ bonds; 2. to become iodinated; and, 3. to react with nitrous acid. The latter reaction in the case of the phenolic group leads through reduction of the primarily formed nitroso derivative to an amine, and further reaction with nitrous acid, to a

typical diazonium salt (161, 179) (Formula VIII). These protein diazo groups resemble simple diazo compounds in their tendency to couple with a great variety of reactive phenolic and diphenolic compounds, yielding brightly colored protein derivatives. Pepsin retained half of its enzymatic activity as the diazo compound and after coupling with naphthylamine. Half of the tyrosine groups of the derivative had become diazotized, i.e., the same number that could readily be acetylated or iodinated (179). Indications have been obtained (55) that the indole groups react similarly with nitrous acid and then couple with amines (49).

Inversely, simple diazo compounds prepared in classical manner from aromatic amines act on this class of reactive —CH groups of proteins (Formula X). This represents the basis of Ehrlich's diazo



test. The imidazole group appears to couple with diazo compounds more readily than does the phenolic; indoles do not give any color under the conditions of the Pauly test as used by Macpherson, but nevertheless may combine with the reagents according to Eagle and Vickers (50). Intensive and repeated treatment with diazo compounds, on the other hand, may introduce considerably greater amounts into proteins than correspond to their histidine and tyrosine contents (19, 20, 123, 99a). It has been suggested that all cyclic residues (123), or all amino and imino groups of proteins (50) may participate in this reaction.

Many protein derivatives have been prepared by the coupling reaction, particularly in connection with immunological studies. The literature on this subject is much too extensive to be quoted here. The interested reader is referred to Landsteiner's excellent book on the specificity of serological reaction (129), and to a recent review article by Kabat (122). The great advantage of this over many other techniques of protein modification lies in the wide vari-

ety of diazo compounds that can be prepared and coupled under the same conditions and with the same protein groups.

It is noteworthy that insulin, when coupled with up to 4 equivalents (per 10^4 g.) of various anionic diazo compounds, retained its activity (186). Recent studies indicate that the phenolic and imidazole groups of insulin and other proteins react to a similar extent with diazo compounds (58a, 62a).

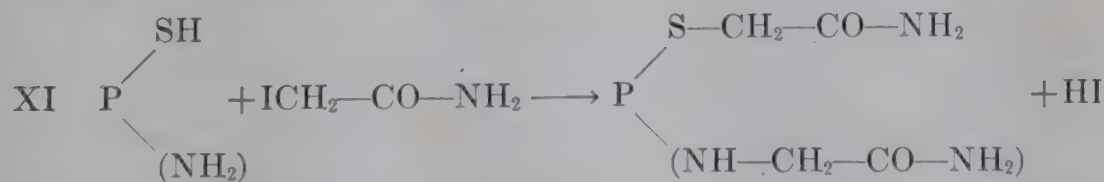
8. Reagents Affecting Sulfhydryl Groups¹¹

The chemical reactions of —SH groups have been more intensively studied than those of all other protein groups. The main stimulus for this work was the observation that the activity of many enzymes depended upon the integrity of certain —SH groups in the molecule. Furthermore, the inertness of —SH groups in many native proteins, in contrast to their great chemical reactivity in denatured proteins and simple mercaptans, stimulated the interest, and the imagination, of many investigators.

Three main classes of reagents have been employed in the study of the —SH groups of enzymes, and proteins generally. These are 1. alkylating agents; 2. oxidizing agents; and, 3. organo-metallic compounds of great affinity for mercaptans.

a. Alkylating agents

Iodoacetic acid or its amide are the main representatives of this class which have been proposed as —SH reagents. These compounds transform —SH groups readily into stable thioethers in neutral or slightly alkaline solution at 0° or room temperature (184) (Formula XI). In some instances —SH groups of native proteins which do not

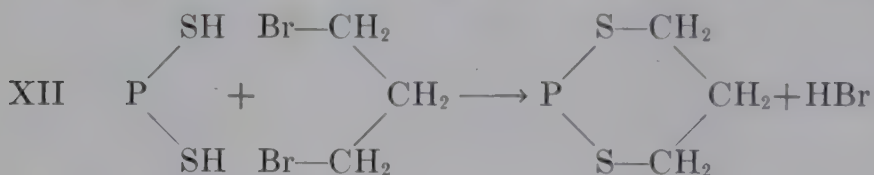


give other typical reactions are able to combine with iodoacetamide under these conditions (8a, b, 3b). However, the action of iodoacetic acid or its amide is not confined to the —SH groups, but may involve some others, such as the amino, indole, or phenolic groups (152, 77a, b, 206, 193, 3b, d) at higher concentrations of the reagent or more alkaline pH. Thus, the combination, or inactivation,

¹¹ Only compounds with more or less claim to being specific —SH reagents will be discussed here. The participation of —SH groups in reactions with acylating agents, alkylating agents, formaldehyde, nitrous acid, etc., has previously been mentioned.

of a protein with iodoacetic acid or its amide cannot be regarded as conclusive evidence for the presence, or essentiality, of —SH groups (10). On the other hand, these compounds can be used in determining the accessible —SH groups of native or denatured proteins, by comparing the cystine plus cysteine content of hydrolysates of the original with that of the alkylated protein. The difference represents the number of —SH groups which have been transformed irreversibly into thioether groups (158a, 180).

Methyl iodide (208, 15) or bromide (131b) and iodoethanol (77, 78) have also been used to alkylate —SH groups. An interesting series of protein derivatives was prepared by Harris and coworkers (175, 75) in the search for moth-proof alkali-resistant wool. These authors reduced the many disulfide bonds of wool to —SH groups (see p. 567) and subsequently treated this product with alkylene dibromides. Thus, they transformed some of the original —S—S— into $\text{—S—(CH}_2\text{)}_n\text{—S—}$ groups, which as thioethers are quite resistant to alkaline and enzymatic hydrolysis (Formula XII).



b. Oxidizing agents

The reducing nature of mercaptans is their most pronounced characteristic. The —SH groups of proteins are often believed to maintain, together with glutathione, the proper intracellular oxidation-reduction potential for a smooth functioning of the enzyme systems. Oxidizing agents would, therefore, appear to be the logical choice as —SH reagents. A variety of oxidants have been used for the purpose of both analysis and protein modification. In recent years, however, criticism has been voiced against the acceptance of this class of agents as specific or reliable detectors of —SH groups (10, 213). This criticism is based in part on the fact that reducing groups other than —SH have been found to occur in some proteins. A further consideration is that the primary oxidation product of —SH groups are —S—S— bonds and that the reaction thus is dependent upon the proximity of pairs of —SH groups (Formula XIVa). Notwithstanding these objections, oxidative methods, generally confirmed and supplemented by other techniques, have supplied the bulk of the quantitative data available on —SH groups in proteins. The inability of a protein to reduce any or all of these re-

agents, on the other hand, cannot be regarded as valid evidence against its containing —SH groups. It has fortunately become general practice to employ more than one type of reagent with any protein not previously studied.

To minimize interference by reducing groups other than —SH, strong oxidizing agents of doubtful specificity are usually not added in excess, but in stepwise increasing amounts, until all —SH groups have become oxidized. The end point of these titrations is the disappearance of the nitroprusside test which, in proteins, appears to be a specific indication of the presence of —SH groups (5). Some of the oxidizing agents and conditions used have been summarized in Table IV. Others, not included, are cystine (158a) and tetrathio-

TABLE IV
OXIDIZING AGENTS SUITABLE FOR DETERMINATION
OF —SH GROUPS^a

Reagents	Medium	Technique	Reference
o-Iodoso benzoate	Guanidine HCl (about 4.5 <i>M</i>)	Iodometric	(104)
Ferricyanide	Guanidine HCl (about 4.5 <i>M</i>)	Titrimetric ^b	(3c, d)
Ferricyanide	Duponol PC (0.1%)	Colorimetric	(3a)
Porphyrindin	Guanidine HCl (6–8 <i>M</i>)	Titrimetric ^b	(89)
Uric acid reagent	Urea (10 <i>M</i>)	Colorimetric	(3c, d)
Iodine in KI (2.5%)	HCl (2%)	Titrimetric	(111b)

^a All except the last reaction occur at pH 6.7 to 7.0 and at room temperature within a few minutes.

^b With nitroprusside as indicator.

nate (3c, d). One equivalent of iodine in *M* potassium iodide at neutrality was found sufficient to oxidize the —SH groups of native egg albumin (3c, d) but those of tobacco mosaic virus protein were affected only when excess iodine was present, and at low iodide concentration. As previously stated, the analysis of all —SH groups of a protein with most reagents has to be preceded by extensive denaturation, iodine being the main exception. The different denaturants which have been found most suitable in conjunction with different oxidants have therefore been included in Table IV. The importance of excluding traces of heavy metals which catalyze autoxidation has often been emphasized (89, 3c, d, etc.).

The neophyte in this particular field of protein chemistry must be strongly cautioned against accepting the listed methods as interchangeably applicable to the determination of —SH groups in any unknown protein. Many of the methods have been applied and tested only in connection with one or a few proteins. In the case of egg albumin approximately the same cysteine content (about 1.0–

1.1 equiv. per 10^4 g.) was found by each of the methods listed.¹² But bovine serum albumin did not reduce ferricyanide (3a), the uric acid reagent, or iodine in acid solution,¹³ yet titrations in guanidine (87) indicate the presence of 0.3 equivalent of sulfhydryl and the mercury binding capacity 0.15 equivalent (i.e., one per mole) (115a). Thus, the determination of the total —SH groups of an unknown protein by any of these methods continues to represent a research problem, rather than a routine analytical procedure.

c. Oxidizable groups other than —SH

Even proteins containing no —SH groups are affected by most oxidizing agents, usually under slightly more drastic conditions than suffice for oxidation of the thiol groups. The phenolic and indole groups appear to be primarily involved in these reactions, particularly with ferricyanide in alkaline solution (158b), and with porphyrindin (23). Under certain conditions the disulfide bonds and the thioether groups of methionine can also be oxidized. Thus, performic acid was found to attack tryptophan, methionine, and cystine (223d). In insulin, which does not contain either of the first two amino acids, it supplied a specific means of oxidizing the cystine to cysteic acid residues (202c). On the other hand, oxidation of the thioether groups may have been the main result when fibrin and fibrinogen were treated with dilute hydrogen peroxide in neutral solution (118). A protein which appears particularly sensitive to oxidation, even by the oxygen of the air, while not containing any —SH groups is the human chorionic gonadotropin (90, 18).

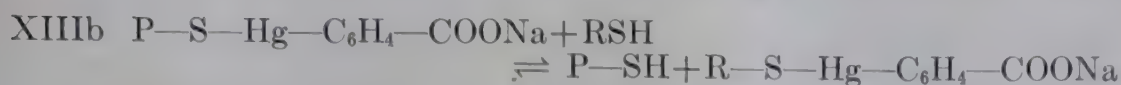
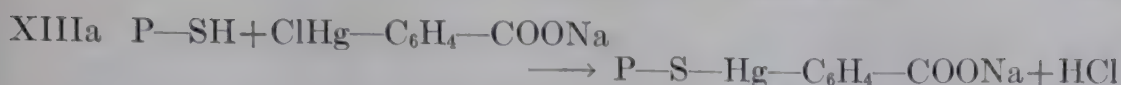
d. Mercaptide-forming compounds

p-Chloromercuribenzoate was first proposed by Hellerman as a specific —SH reagent (102, 103) and has since been extensively used. Various organic arsenicals (10, 213), as well as other mercuric compounds (102, 103, 235) appear to act similarly, though not quite so effectively (10).¹⁴ The advantages of this class of reagents are 1. they react with single, rather than with pairs of —SH groups (Formula XIIIa); 2. they react with groups which appear masked to

¹² The variations from this value reported in the literature (165) are probably due to the heterogeneity of egg albumin.

¹³ Unpublished experiments by Fraenkel-Conrat and C. H. Li that are contrary to Hess and Sullivan's findings (111b).

¹⁴ The action of war gases of the Lewisite type (chlorovinyl dichloroarsine) is attributed to their tendency to combine with the —SH groups of the enzyme systems (222). Many lachrimators have also recently been found to react with these groups, probably by alkylation (147a). The mechanism of action of chlorpierin on —SH groups is unknown (67a).



most oxidizing agents; 3. they appear highly selective in combining with no protein groups other than —SH; and, 4. their combination with —SH groups is readily reversible by the addition of an excess of a simple mercaptan (Formula XIIIb). (1, 2 dimercaptopropanol, British Antilewisite (22) has proven most effective for this purpose.) The latter phenomenon is of great advantage in the case of biologically active proteins, and it is in the study of enzymes that these reagents have found their primary field of application. Thus, the many enzymes requiring —SH groups for their activity can be reversibly inactivated and reactivated by the addition of *p*-chloromercuribenzoate, and mercaptans, respectively. *p*-Chloromercuribenzoate has also been used for the determination of the total —SH content of denatured proteins (3c, 148a); a titration technique with nitroprusside as the end point indicator was employed.

e. Reactivity of —SH groups of enzymes

The great interest in —SH tests, in general, is aimed not so much at finding reagents and conditions suitable for quantitative analysis, but at a differentiation between the various states of reactivity of the —SH groups. The work of Balls and Lineweaver (8a, b), Hellerman (103), Barron and Singer (10, 213), Winnick and Greenberg (86, 239), and others has demonstrated that there exists a scale of reactivity for the —SH groups of certain enzymes, and that anywhere along that scale there may be —SH groups essential for the activity of the enzyme, while others may be modified without interference with the activity.

Hellerman and coworkers (103, confirmed in 45b) have classified the —SH groups of native urease into: a. those readily accessible to a variety of reagents; and, b. those accessible only to *p*-chloromercuribenzoate, but not to the oxidizing or alkylating agents used. Yet a third group (three times as many as in a. or b.), which we might classify as c. becomes reactive only upon denaturation. Papain appears to possess no —SH groups corresponding to the a-group of urease. One —SH group, however, appears somewhat more reactive than the b-group of urease, being affected by cystine or iodacetamide, but not, while the protein is in the native state, by iodine, porphyrindin, or nitroprusside (8a, b). In both enzymes the

activity is dependent upon the b-type of —SH groups being in the reduced state. Some intracellular animal proteinases (cathepsins) appear to belong to the same class (72, 148a), as well as bromelin and a number of plant proteinases which have been described by Greenberg and Winnick (86, 239). All but one (solanain) resembled papain in being reversibly activated and inactivated by a variety of reducing and oxidizing agents. An interesting study with crystalline ficin (238) has revealed that this enzyme, like pure papain, can be obtained in an active state, yet giving a negative nitroprusside test. Its —SH groups, apparently of the b-type, are, however, very sensitive to autoxidation. Thus, the enzyme, when freed from simple mercaptans, is readily inactivated by air (also by permanganate), but can be reactivated by reducing agents. It would appear that the protective role which the a-type of —SH groups plays in the case of urease is taken over by low-molecular natural activators (thiols) in the case of ficin and papain.¹⁵

The adenosine triphosphatase activity of myosin was similarly found to be dependent upon masked —SH groups, reactive toward *p*-chloromercuribenzoate but not toward oxidizing agents unless the protein was denatured (213a). This protein shows the singular property that its free and reactive —SH groups appear masked in the presence of varying concentrations of such substances as glycine and ammonium chloride (88).

Weill and Caldwell (235) have shown that the activity of β -amylase, in contrast to that of pancreatic amylase (27, 140a), depends upon the presence of —SH groups. Barron and Singer (10, 213) have spent considerable effort on a systematic investigation of a great number of enzymes in regard to their possession of essential and reactive —SH groups. These authors have relied mainly on reversible inactivation by *p*-chloromercuribenzoate as the criterion of the presence of such groups. The studies of the essentiality of —SH groups for the activity of enzymes have been reviewed by Singer (212), who also discussed critically the meaning of the term "essential groups" (212a).

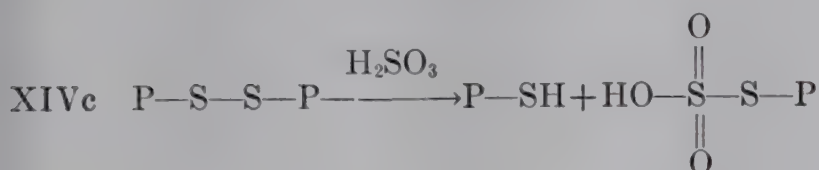
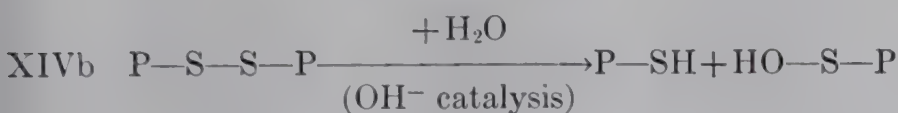
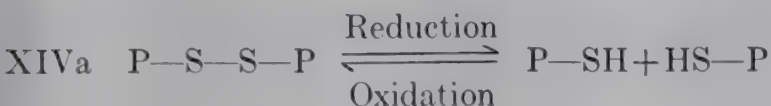
The great differences in the behavior of —SH groups are not peculiar to enzymes but occur also in other proteins. Thus, the tobacco mosaic virus protein is remarkable in that it contains masked —SH groups which may be oxidized without interference with its infectivity (4a).

¹⁵ The mechanism of oxygen poisoning has been intensively studied in recent years and indications have been obtained that the oxidation of —SH enzymes at excessive oxygen pressures may be responsible (98).

9. Reagents Affecting Disulfide Bonds

Reducing Agents

The —S—S— bonds of cystine play an important role in maintaining the molecular architecture of many proteins, particularly of those rich in cystine, e.g., the keratins and insulin. The accessible disulfide bonds of all proteins of this type are affected by reducing



agents (228, 77, 120a), alkali (204, 43, 160), sulfite (37, 154b, 139), or cyanide (232, 43), according to formulas XIVa to XIVd. These reactions have been intensively studied in recent years by several groups of workers, with particular reference to the keratins of hair and wool (204a, 43, 139, 223b, 175, 75). It appears that a certain proportion of the disulfide bonds in these proteins, and also in insulin, are characterized by being quite susceptible to the above four reagents, in contrast to other native proteins which are generally quite resistant to their action. ⁶

Various reagents have been used to reduce the —S—S— bonds according to formula XIVa. The most specific and effective appear to be sodium sulfide and sulfhydryl compounds. Thioglycolic acid, mercaptoethanol, cysteine, or glutathione are generally used in excess, in neutral or slightly alkaline solution if extensive reduction is intended (77, 175, 120, 228, 240, 56a).¹⁶

Sodium in liquid ammonia (190, 190a) and sodium amalgam (69, 225) have also been used, as well as tin or zinc in acid. None of

¹⁶ Fibrous proteins rich in disulfide bonds are not readily dissolved by reducing agents unless alkali, detergents, or other denaturing agents are simultaneously used to break the hydrogen bonds holding the molecules in fibrous orientation (183, 77, 120). While there is little doubt that the thiol reagents primarily reduce —S—S— bonds, indications have been obtained that at high concentration they may cause the formation of some unknown reducing group in the treated protein (56a).

these reagents is without effect on other protein groups. Catalytic reduction did not appear to affect the disulfide bonds of insulin (94), although Freudenberg found the hormone to become inactivated by such treatment (69).

Reduction of proteins has sometimes been performed not with the aim of splitting disulfide bonds, but with that of splitting off the iodine from iodinated phenolic groups (94), or of removing the carbobenzoxy group from amino groups. Catalytic reduction has been found most suitable for the latter (94, 38, 39, 116).

Many biologically active proteins have been shown to be inactivated by reduction of their disulfide bonds. Some, like insulin (228, 240, 220a, 236) and crotoxin (216) contain readily reducible bonds, the splitting of which results in inactivation. Others, like the pituitary lactogenic hormone (56a, 66b),¹⁷ may be partially reduced without being inactivated. Another group of hormones are only inactivated by quite intensive reducing treatment, presumably because their —S—S— bonds are less reactive than those of insulin (66a). A fourth class of biologically active proteins may retain their activity after extensive reduction of their disulfide bonds, although the extent of reduction has in no case been quantitatively demonstrated (208, 215).

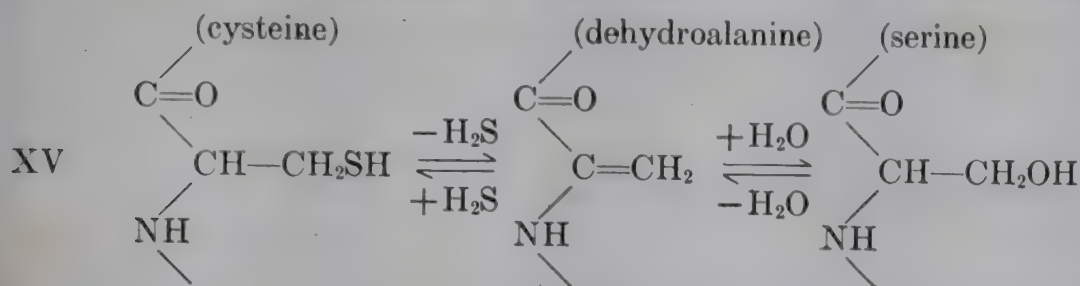
Because of the autoxidizability and reactivity of the groups formed in the four disulfide splitting reactions (Formula XIV), the isolated products, after removal of the reagent, do not generally conform to the simple stoichiometric relationship indicated in the above scheme. Reaction a) and c) have been shown to be reversible, but if an appreciable number of —S—S— bonds are broken, the probability of the original pairs becoming reunited is small.¹⁸ Further, —SH groups formed in reactions b)-d) may, under certain conditions, become reoxidized to —S—S— groups more readily than they enter into the reverse phase of the reaction through which they had originated.

The sulfenic acid groups (—S—OH) (Formula XIVb) believed to be formed with alkali are quite unstable and may undergo a variety of reactions with other protein groups. These reactions have supplied a wide field for research and speculation (43, 139, 204a, 223a,

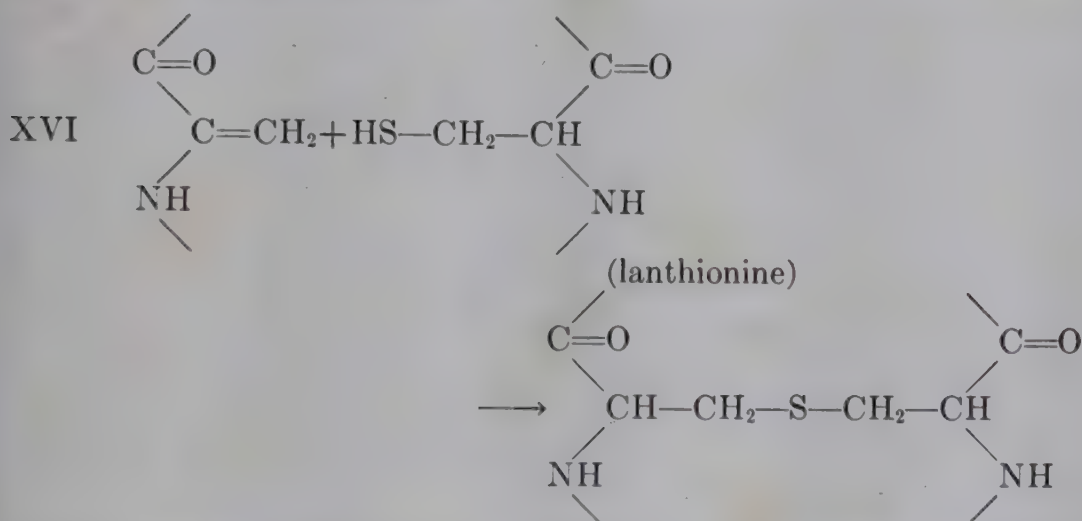
¹⁷ The lactogenic hormone resembles insulin and other disulfide proteins in being highly insoluble in the reduced state near neutrality. It must be solubilized by high concentrations of urea before its biological activity can be demonstrated (56a, 66b). As previously stated, the insolubility of such artificial —SH proteins is indicative of their highly aggregated state, as proven in a quantitative manner for reduced insulin (156).

¹⁸ It is therefore not surprising that attempts to regenerate the biological activities of reduced proteins by means of reoxidation have almost invariably not been successful. Freudenberg's studies represent an exception (71, 70).

160). An intermediate which is almost certainly formed from cysteine and cystine, as well as from serine residues, under favorable conditions of alkalinity, is dehydroalanine (Formula XV). An addition reaction between dehydroalanine and cysteine residues can explain the



formation of lanthionine from proteins rich in cysteine upon treatment with alkali (Formula XVI) (115, 227). Up to half of the cysteine of wool may yield lanthionine under suitable conditions of alkali or cyanide treatment (43, 139). Addition of hydrogen sulfide



(Formula XV) or benzyl mercaptan to alkali-treated proteins has led to the transformation of serine, presumably through dehydroalanine, to cysteine or benzylcysteine residues (167a, b). Other end products of the action of alkali on disulfide bonds appear to be elementary sulfur (204a), and cysteic acid (40). Others may yet be identified.

Technique of extensive reduction of disulfide proteins (56a)

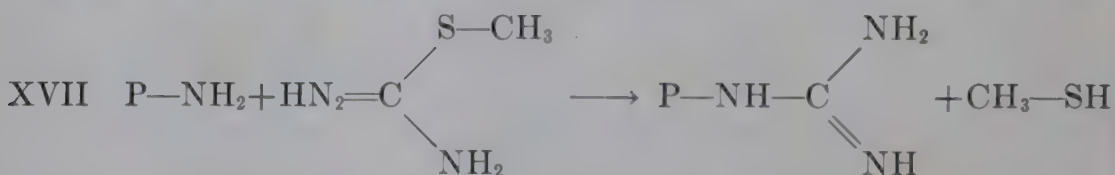
To the protein in 0.1% or more concentrated solution buffered with phosphate to pH 7.6 to 8.0 is added a 40 fold amount of cysteine hydrochloride previously neutralized with an equivalent amount of sodium carbonate. The reaction mixture is held at room temperature for two days. Thioglycol or sodium thioglycolate may be used in preference to cysteine for most purposes. Insulin is extensively reduced by much lower thiol concentrations (156).

10. Interconversion of Protein Groups

The development of efficient methods to achieve the transformation of one type of protein group into another unfortunately has not kept pace with the urgent need for them. The transformations that can be achieved generally only involve a fraction of the possible groups and are often accompanied by other modifications or degradation.

a. Guanidylation, deguanidylation

The replacement of amino by guanidyl groups has repeatedly been achieved. Cyanamid has been used, but S-methylisothiurea or O-methylisourea appear to be the best reagents. When the former was permitted to act on casein at room temperature for three weeks in 3.5% ammonia, the amino nitrogen of the protein largely disappeared (Formula XVII). No other analyses, however,



established the specificity of the reaction (207). Christensen (36) and Roche (191) have since demonstrated the newly formed guanidyl groups by means of the Sakaguchi reaction. O-methylisourea appears to react somewhat more readily (at pH 10 and 0°) (115a).

The opposite reaction may be brought about by treatment with alkali, but probably not without breakdown of peptide and amide bonds (128a, 198c).

Boiling acetic anhydride transforms arginine into acetyl ornithine residues, but it is not certain whether any arginine residues or only those which are terminal members of the peptide chain react in this manner (17, 46). Hypochlorite in strongly alkaline solution destroys the guanidyl groups, but the nature of the final product has not been elucidated. Tyrosine, tryptophan, and histidine are also affected (198b). Collagen has been partly dearginated by a similar technique (112).

b. Amidation, deamidation

Treatment of proteins with nitrous acid in the presence of mineral acid transforms all amide into carboxyl groups (61). But deamination and extensive destruction of the guanidyl groups occurs at the same time. Attempts to hydrolyze the amide bonds without

any breakdown of the peptide chain appear never to have been successful (211).

The opposite reaction, leading from the acid to the amide, may be more easily accomplished. A microbiologically synthesized glutamic acid polymer has been transformed to the polyester, and that to the amide, by acid-methanol (see section on esterification) followed by liquid ammonia treatment (62, 64).

Another interesting transformation, that of the aliphatic hydroxyl to the sulfhydryl group, has been previously discussed (167).

11. Miscellaneous Reactions

Nitration of the phenolic groups results from treatment of proteins with nitric acid at room temperature; the reaction is accompanied by some increase in amino nitrogen (136, 242). The indole rings are also nitrated under these conditions (12). Tetranitromethane appears to achieve the substitution of the phenolic group under milder conditions (242); unfortunately, this interesting reaction has not yet been extensively studied. Nitration of the guanidyl groups has been achieved in protamines and proteins by means of a mixture of fuming sulfuric and nitric acids at 0° (128b).

Ninhydrin has been proposed as a preparative means of abolishing the amino groups, but the mechanism and the specificity of the reaction was not studied in detail (48, 51). The effect of nitrogen trichloride of rendering certain proteins, particularly wheat gluten, toxic for dogs has recently been shown to be due to its action on some methionine residues (12c, d).

Non-proteolytic enzymes have at times been investigated in the hope that they might supply specific means of attack on certain side chains of proteins. Tyrosinase, it was recently reported, can oxidize part of the tyrosine residues of some proteins (214b). This reaction has been denied (50a) and reaffirmed (214c). Phosphatases from frog eggs (96) and mammalian tissues (51a) were shown to split the serine phosphate ester bonds of phosphorproteins (96). But the specificity of most enzymes is such that it does not favor a direct action on protein groups, as exemplified by arginase which requires, besides the guanidyl, a free carboxyl group (53).

12. Conclusions

A brief discussion of protein modification as a whole may serve to illustrate the more general aspects and indicate some uses that this branch of chemistry may serve. The following main types of derivatives can be prepared by the indicated reactions:

1. The polar groups can be increased in number by 1, 2-epoxides, formaldehyde, phosphorylation, or coupling.

2. They can be decreased in number, by acylating and alkylating agents.

3. Acid groups can be introduced¹⁹ by carbon suboxide, phthalic or succinic anhydride, sulfation, phosphorylation, coupling, or the combined action of formaldehyde and amino acids.

4. Basic groups can be removed, and the protein thus rendered more acidic, by acylation and deamination. Iodination and nitration increases the acidity of the phenolic groups.

5. Acid groups can be removed and the protein thus rendered more basic by esterification with diazo methane, methyl alcohol, 1,2-epoxides, or mustard gas.

Besides these effects on the polar groups, certain reagents may affect the molecular weight or shape, through the introduction of intermolecular and intramolecular crosslinks. Such reagents are formaldehyde (63a, 65) (Formula IXb, c), carbon suboxide (173) (Formula IIb), possibly quinone (223a, 67), and carbon disulfide (67). Oxidation of free —SH groups or condensation of various split products of disulfide bonds may have similar effects. All bifunctional reagents (e.g., glyoxal, diisocyanates, dibromides, quinones, mustard gas) would be expected to introduce crosslinks. This appears to have been demonstrated only in one instance (175). Dry heat may also crosslink peptide chains through the formation of new imide bonds between carboxyl and amino groups. Decreases in acid and basic groups (150, 144) and increases in strength of fibers (144) have been demonstrated. The involvement of the ϵ -amino group of lysine, in particular, is evident from nutritional studies of heated proteins (210, 85, 97).

Inversely, splitting of —S—S— bonds by a variety of agents decreases the number of natural crosslinks, and thus the molecular weight (84, 156). The reactive groups formed may enter into new crosslinking combinations, both through primary and secondary bonds, as illustrated by the formation of lanthionine (115, 227) and the aggregation of reduced insulin (156).

The preceding discussion of the selectivity, or lack of selectivity, of various reagents in regard to the different protein groups is summarized in Table III. Some applications of these methods in the search for the groups essential for the biological activities of

¹⁹ No reliable methods to introduce basic groups have been described. Treatment with ethylene imine or the nitrogen mustards appears to achieve this end. Guanidylation introduces a stronger in place of a weaker basic group.

proteins are listed in Table II. For the sake of brevity, only experiments supported by analytical evidence have been included. Thus, the fact that most of the conclusions are based on the use of several reagents is not evident from the table; nor does it include many valuable papers on other biologically active proteins, the essential groups of which were elucidated by means of a variety of reagents instead of by a thorough analytical study of the effect of one reagent. A more complete review of this subject is given by Herriott (108) and Olcott and Fraenkel-Conrat (172).

One noteworthy fact appears from these studies. While amino, disulfide, and thiol groups may or may not be essential for the activities of the various proteins, some phenolic groups were found essential in every case where they were investigated.

IV. METHODS OF ANALYSIS FOR PROTEIN GROUPS

1. Amino Groups

The amino groups of proteins are readily determined by Van Slyke's manometric or volumetric nitrous acid method. The reaction periods usually chosen are 15 minutes for the manometric and 20-30 minutes for the volumetric apparatus (226). Consistent results can be obtained even with water insoluble proteins, when introduced as fine powders. Various modifications of the manometric apparatus have facilitated analyses of insoluble materials (197, 47). Most soluble proteins precipitate during the test and low values may result if gross coagula are formed in this process.

The results obtained with this technique do not represent by themselves accurate determinations of the absolute number of amino groups of proteins, since the rapidly completed liberation of nitrogen from the terminal alpha amino groups of the peptide chains and the only slightly slower reaction of the ϵ -amino groups of lysine, are accompanied by a very slow and continuous liberation of nitrogen from the guanidyl groups of arginine (197, 137, 47). Terminal glycine also gives more than one mole of nitrogen (202a, 35),²⁰ and indications have been obtained that the indole residues may liberate traces of nitrogen during the reaction with nitrous acid (203). The phenolic groups are transformed to diazo compounds (161, 179) and may liberate nitrogen if strong light falls on the

²⁰ Viscontini (230) has recently shown that nitrous acid transforms di- and tri-glycine into oxalyl glycine and oxalyl diglycine. This oxidation serves well to explain the excess nitrogen formed by glycine in terminal position in the peptide chain (202a).

reaction chamber (56b). To obtain accurate, absolute amino nitrogen values, errors due to these slow reactions can be minimized by analyzing the protein for various time periods and extrapolating the flat part of the resulting curves to zero time (197, 137, 47). For routine or comparative analyses, however, a standard reaction period of 15 minutes yields adequate values. With most proteins, these correspond to 105–111% of the correct value; with insulin about 115% of the true value is found, because of the terminal position of glycine residues in this protein (202a).

It is not generally recognized, however, that many simple chemicals seriously interfere with the Van Slyke amino nitrogen determinations. Small amounts of ethyl alcohol cause serious errors, as well as many reducing substances (32), phenols (56b), and malonic acid or its esters (194). Ammonium ions also prevent accurate analyses (116a, 178) by reacting slowly with nitrous acid. A rather complete discussion of interfering substances has been given by Richardson (189a).

Sørensen's formol titration method has also often been applied to proteins, although the exact significance of the values obtained has not always been clear. Recent work by Cannan and his coworkers (28) has indicated that the ϵ -amino groups may be titrated by adjusting the protein solution to pH 8.5, and titrating it to this same point after addition of formaldehyde. The α -amino groups and some of the imidazole groups are included in the titration if the original solution is adjusted to pH 6.5, instead of to pH 8.5.

A colorimetric method based on the use of ninhydrin has at times been employed for the determination of the amino groups of proteins (93). It appears to give rather consistent values which, depending upon impurities in the ninhydrin used, may be as much as 15% lower than those obtained by the Van Slyke method. Thus, the colorimetric method appears more suitable for comparative work than for absolute amino nitrogen determinations in proteins.

For details concerning the use of the Van Slyke apparatus, the reader is referred to laboratory manuals (178). The ninhydrin method will be described in detail because of its simplicity.

Technique of ninhydrin amino-nitrogen analysis (93)

To 5 to 20 mg. protein (containing 0.01–0.08 mg. $\text{NH}_2\text{-N}$) dissolved or suspended in 1 ml. water is added 1 ml. 10% aqueous pyridine and 1 ml. of a freshly prepared 2% solution of ninhydrin (preferably recrystallized). The mixture is loosely stoppered and heated

in a boiling water bath for 20 minutes. It is then diluted to 100 ml. with water and the color read at or near 565 $m\mu$. A standard curve is prepared with alanine in the same manner.

2. Other Basic and Carboxylic Acid Groups

No simple methods appear to be available for the determination of guanidyl and imidazole groups in intact proteins. The Sakaguchi test serves as a useful qualitative indication for the presence of guanidyl groups (198a, 234, 191); no qualitative test specific for the histidine residues appears to exist, although the Pauly diazo test, as applied by Macpherson is given much more strongly by histidine than by tyrosine.

The careful work of Cannan (28) has established the possibility of determining each of the basic and the carboxylic groups by a critical interpretation of electrometric titration curves obtained with and without addition of formaldehyde. Most of the values obtained are in remarkably good agreement with analytical results for the corresponding amino acids obtained on hydrolysates by Chibnall and others (35) (see Table I). Thus, it is well demonstrated that the basic and acidic groups are not masked or cross-linked in the native protein. With the exception of the recent work of Herriott and coworkers (109), titration methods have not been applied in a routine fashion as analytical tools.

Analytical methods exist, however, for the sum of the basic groups, i.e., the α - and ϵ -amino, guanidyl and imidazole groups. These can readily be determined by the amounts of metaphosphoric acid (177) or of an acidic dye (60) bound and precipitated with the protein in acid solution. A similar method with a basic dye in alkaline solution permits the determination of the sum of the acid group i.e., the carboxyl, phenol, and thiol groups (60). The theoretical basis of these methods, as well as that for the interpretation of titration curves will be dealt with in another chapter. Here it may suffice to point out the availability of simple methods for the routine determination of the polar groups of proteins based on colorimetry and posing no difficulties of interpretation.

The number of carboxylic (not total) acid groups occurring in soluble proteins has been found to be closely approached by their ability to combine with methanol in the presence of 0.1 *N* hydrochloric acid at room temperature (see section on esterification). Thus, methoxyl analyses may supply rather good approximations of the carboxyl groups of many proteins (64).

3. Phenolic and Indole Groups

The phenolic groups of proteins are generally estimated by means of the Folin phenol reagent. If the alkaline buffer and the acidic reagent are added simultaneously, the reaction occurs under the gentlest conditions and does not usually entail protein denaturation. However, the values obtained are not of absolute but only of comparative value, for several reasons: 1. Both tyrosine and tryptophan residues contribute to the chromogenic value of a protein, besides any other reactive reducing groups (e.g., —SH groups and heme); 2. The chromogenic value of peptides of tyrosine and tryptophan is lower than that of the free amino acids; thus, the values obtained for a protein can never be expected to correspond to its total tyrosine and tryptophan content (224a, 157b); 3. Most native proteins give values which fall considerably short even of those obtained for simple peptides (107a, c). A range of different values can be obtained depending upon the extent of denaturation and varying for various native proteins from 42 to 73% of that corresponding to their true tyrosine and tryptophan content; 4. The only requirement for a positive phenol test appears to be the free hydroxyl group. Substituents on one or both orthopositions such as —OH, —I, and —CH₂OH, do not forestall the development of color, although they may affect its intensity (107d).

Notwithstanding these weaknesses, the Folin reagent has been extensively used and has certainly supplied much valuable comparative data. It has proven most useful when applied according to the procedure of Herriott (107a), particularly for the determination of alkali-labile phenolic esters. The reagent can also be used for the determination of the total tyrosine and tryptophan content of proteins or protein derivatives, if these are hydrolyzed by alkali or preferably enzymatically prior to analysis.

In a qualitative manner, the Millon test has often been used to ascertain the participation of the phenolic groups in protein reactions of biological interest. In contrast to the Folin test, the Millon reagent distinguishes between unreacted phenolic groups and those substituted not only on the oxygen but also on the orthocarbon atoms. (Monoiodotyrosine, however, gives a positive test.) The conditions of this reaction are such as not to be applicable to native proteins and to the more labile derivatives.

A recent reinvestigation of the possible usefulness of ultraviolet spectrophotometry (81a) may provide a method for the determination of the tyrosine and tryptophan residues of unhydrolyzed pro-

teins dissolved in 0.1 *N* alkali. For the simpler problem of determining tryptophan in the absence of tyrosine, ultraviolet spectrophotometry has proven useful in the case of gramicidin dissolved in alcohol, but modification of the indole rings did not change the character of the absorption spectrum (63).

Technique of Folin phenol plus indole analysis (107a)

An amount of protein containing 0.05–0.3 mg. of tyrosine plus tryptophan is dissolved in water (or dilute NaCl, or a minimum of acid or alkali), made up to 8.5 ml. and treated simultaneously with 2.5 ml. buffer (60 ml. 0.5 *M* Na₂HPO₄ and *N* NaOH to 100 ml.) and 1.5 ml. 1:3 diluted Folin phenol reagent. The solutions are held for one hour at 40°, permitted to cool and read colorimetrically with a purplish-blue filter; standard curves may be prepared from equivalent amounts of tyrosine or tryptophan.

To recover the free phenolic groups from labile esters, e.g., acetates (Herriott's pH 11 method), water is added to only 5.5. ml., followed by 1.5 ml. 0.1 *N* NaOH. After 15 minutes' standing at room temperature, 1.5 ml. of 0.1 *N* HCl is added, followed by buffer and reagent as above.

4. Other Protein Groups

The methods proposed for analysis of sulfhydryl groups, and the difficulties arising from their masked state, have been previously discussed. There appears to be no method available which is equally applicable to all proteins known or believed to contain —SH. Titrations in concentrated guanidine hydrochloride (3a, c, d), with nitroprusside as end point indicator are easily performed and often give reliable results. Under such conditions, however, bovine serum albumin reacts much more slowly than do egg albumin and β -lactoglobulin. The iodine titration method of Hess and Sullivan (111b), and the application of the Folin uric acid reagent according to Anson (3c, d) give similar values with the above method in the case of egg albumin, but unreliable results with the other of the above-named —SH proteins.

Disulfide groups are usually determined as sulfhydryl groups after reduction (158a, b). Their non-reactivity in many native proteins raises the same problems which complicate the —SH analysis.

Protein amide groups can only be determined concomitant with at least partial hydrolysis of the protein. Treatment of proteins with nitrous acid in strong mineral acid at room temperature appears to cause little if any hydrolysis of the peptide chain, but lib-

erates the total amino and amide and about 90% of the guanidyl nitrogen within 24 hours (61).

For aliphatic hydroxyl groups there is no simple method of analysis available. The periodate oxidation method can only be applied to serine and threonine after complete hydrolytic liberation.²¹ The amount of sulfate sulfur bound by proteins upon treatment with cold concentrated sulfuric acid may represent an approximate measure of their hydroxyamino acid content (187).

²¹ Hydroxylysine, which occurs in small amounts in gelatin and some other proteins, yields formaldehyde directly upon treatment of the protein with periodate (45). The finding that no formaldehyde was formed from sericin is interpreted as proof that none of the serine, which comprises about 35% of this protein, occurs terminally on the peptide chains.

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Chapter X

NUTRITIONAL APPLICATIONS OF THE AMINO ACIDS

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I. NUTRITIONAL STATUS OF THE AMINO ACIDS

UP TO COMPARATIVELY recently, the knowledge of the requirement for the amino acids in the diet was limited to information obtained with the young rat. This animal was able to grow when receiving only nine amino acids: namely, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine; and grew significantly better when arginine was also provided. This latter response indicated an insufficient synthesis of arginine. These ten amino acids were called "indispensable" for the growing rat, while the remaining known amino acids were called "dispensable" (302). However, there was no implication that the dietarily dispensable amino acids are of little or no value.

In spite of cautions issued (302) there has been a general tendency to assume that the above classification has very broad application. That this is not necessarily true is shown by the fact that the chick obtains all its needed arginine from the diet and also some of its glycine; the young mouse or adult rat does not require arginine; the adult human does not require histidine. Ruminants probably do not have a critical dietary need for any amino acid, because of their extensive and productive system of micro-organisms which can build, from simple sources of nitrogen, the amino acids and proteins that are ultimately utilized by the ruminant. Very young ruminants, in which the system of micro-organisms is not yet well established, do require proteins of a high degree of completeness, *e.g.*, milk proteins.

The most recent analyses of body tissue show that practically all of the dispensable amino acids are present. These amino acids may comprise 40% or more of the tissue protein. It is obvious that the "dispensable" amino acids are physiologically indispensable to the formation of the characteristic proteins of the animal. If not obtained in the diet they must be synthesized *in toto* from other sources of nitrogen and carbon. For example, when so compelled, the animal will manufacture all its cystine from methionine and serine, or its tyrosine from phenylalanine. It can readily be shown, however, that when methionine or phenylalanine are at marginal (but not severely deficient) levels, dietary cystine or tyrosine, respectively, have growth-promoting action (302). While further specific examples are lacking, it is rather likely that the other "dispensable" amino acids have some "sparing action" of a general nature.

Very recently it has been shown that with a minimum allowance

of indispensable amino acids, and no dispensable amino acids in the diet, the rat is able to utilize ammonium compounds for growth, evidently through a facilitated amino acid synthesis (233a). Certain dispensable amino acids and urea can also be utilized for this purpose (308a).

Remarkable physiological roles of certain of the dispensable amino acids have been uncovered. For example, glycine is a vital substance in the synthesis of creatine, which, in a phosphorylated form, participates in the energy metabolism of muscle. Serine furnishes the carbon chain for the *in vivo* synthesis of cystine and choline, and of tryptophan by certain micro-organisms. Glutamic acid in the form of glutamine is intimately connected with the processes of ammonia transport and urea formation. Glutamic acid is an integral part of the vitamins of the folic acid group. Together with glycine and cystine, glutamic acid forms a tripeptide, glutathione, of wide occurrence in living tissue. Tyrosine is closely related to the essential principle of the thyroid gland, thyroxine, and possibly to other hormones as a precursor.

The concept of the indispensable amino acids had a great value in focussing attention on the nutritionally most critical amino acids, and in stimulating the development of methods for their determination in food proteins, as well as studies of their requirement by several species. However, the division of the amino acids into indispensable and dispensable groups now imparts such an inadequate impression of modern knowledge that there is a real question as to whether this separation should be any longer emphasized.

1. Individual Amino Acids

The nutritional status of the amino acids has been extensively reviewed (302, 197, 26, 380, 88, 116). The biochemical applications of the amino acids cannot be presented without first bringing their nutritional status up-to-date.

Arginine

Arginine was found to be required in the diet of the rat only to accelerate the rate of growth (302, 76). The mouse grows moderately well without arginine (40). Arginine does not seem to be required for maintenance of nitrogen balance in the adult rat (379, 87), dog (308), or human (303, 304a, 191). In the last species, an arginine deficiency in the diet is followed by a markedly retarded spermatogenesis (191). The growing rat on an arginine-free diet

shows anatomical abnormalities in the testes (7). Of all body tissues, the sperm cells might be expected to be the first to be damaged by an effective arginine deficiency since sperm proteins are extremely rich in arginine. Arginine is also regarded as essential for blood protein regeneration in the dog (203).

Casein is an inadequate source of arginine for the chick (46, 40a, 220). The young chick is unable to synthesize arginine even when furnished ornithine (220). However, the chick utilizes citrulline, apparently by converting it to arginine (217), as is done by the rat (79). On the other hand, the pigeon seems to be unable to convert citrulline to arginine (67). Obviously, in the fowl, the Krebs ornithine cycle cannot operate. Deficiency of arginine in the diet of the chick results not only in poor growth or actual loss of weight, but also in a profound weakness and a reduced muscle creatine content (175, 31). The arginine requirement of the young turkey is higher than that of the chick (224). It has long been known that the fowl can mobilize ornithine for the detoxification of benzoic acid, which is excreted as dibenzoyl ornithine (ornithuric acid). An exactly analogous derivative is formed when an excessive level of nicotinic acid is fed to the chick (113).

The unnatural isomer, D-arginine, seems to follow the same metabolic path as the L-form in man. Human liver arginase yields equivalent amounts of urea from both D- and L-arginine. However, rat liver arginase does not split D-arginine (15). Accordingly, the latter may not be well utilized by the rat. Argininic acid also is poorly utilized, if at all, by the young rat (76).

Histidine

Histidine is required for growth of the rat (302, 7, 170), mouse (358) and chick (31, 220). The status of histidine for the adult rat is uncertain, since it has been found necessary in a mixture of nine amino acids which, jointly, will maintain a positive nitrogen balance (379), and in an amino acid mixture which will permit weight maintenance (7). Other investigators (87) have reported that only seven amino acids, not including histidine, are needed to replace the loss of nitrogen in the adult rat. The removal of histidine from the diet of the adult dog is followed by large nitrogen losses (308). In adult man, however, histidine appears to be entirely dispensable for maintenance of nitrogen equilibrium (307, 304a, 15). This may not hold true for pathological cases, since a patient with ulcerative colitis entered a negative nitrogen balance when deprived of histidine (246).

Rats will grow and incorporate L-histidine in the protein of tissues when receiving a histidine-deficient basal diet, supplemented with D-histidine, thus demonstrating an ability to invert the unnatural antipode (103). D-histidine is utilized for growth by the mouse, but less effectively than the natural form (358). D-histidine may not be metabolized when given to adult humans, since an excess of histidine was found in the urine equivalent to the amount of D-histidine ingested, and the excreted isomer was identified by isolation (11).

Lysine

Lysine is required in the diet of the growing rat (302), chick (37, 159), and mouse (50, 358). D-lysine is entirely incapable of replacing L-lysine for growth purposes in the rat (55) and in contrast to natural lysine, the D-isomer is excreted largely unchanged (276, 293). D-lysine does not accelerate the growth of mice on a lysine-deficient diet (358). Neither the carbon chain nor the nitrogen of isotopically labeled D-lysine was found in the L-lysine of rat tissues after feeding (293). The ϵ -hydroxy analogue of lysine is also unable to replace lysine in the diet of the rat (147).

It has not been possible to demonstrate the synthesis of lysine in higher animals. While it can be deaminated *in vivo* and thus provide nitrogen for the synthesis of other amino acids, lysine is not regenerated, as is indicated by the results of isotopic tracer studies in which both the carbon chain and the α -amino nitrogen atom of lysine were labeled (367). L-lysine may, perhaps, be regarded as the most specifically indispensable of the amino acids, since all of the others can be replaced by closely related compounds, or by the optical isomers.

In view of the complete absence of lysine synthesis in experimental animals, it is peculiar that indications should be obtained of its dispensability for maintenance of nitrogen balance in adult rats (87). This report may possibly be explained by more recent studies which show that the maintenance requirement is comparatively small (277, 265). The adult rat requires only 16 mg. of lysine per day to maintain its body weight; this is about one-sixth the amount needed for optimal growth (277). Nitrogen losses follow the removal of lysine from amino acid mixtures which will maintain a positive nitrogen balance in young men (303).

Lysine may be destroyed in casein by treating the protein with nitrous acid, which attacks the free ϵ -amino group of lysine (120). Casein so treated and fed as the source of protein to young rats promotes the development of an anemia which can be cured by

the subsequent addition of sufficient lysine to the diet (187, 146). It has been reported that the ϵ -hydroxy analogue of lysine is found in nitrous acid-treated casein, and is a positive cause of anemia in rats (286). This is probably another example of metabolic displacement, or of an "antimetabolite." Young rats on a lysine-deficient diet develop a progressive vascularization (capillary invasion) of the cornea which is reversed by restoring the lysine to the diet (185).

Nitrous acid-treated and subsequently acid-hydrolyzed casein will not maintain nitrogen balance in human subjects unless both tryptophan and lysine are added (13). The subjects on this lysine-deficient diet at times experience nausea, dizziness and hypersensitivity to metallic sounds. There is a very general increase in the urinary excretion of organic acids. These symptoms are not observed with like deficiencies of tryptophan and methionine; hence, they may be a specific syndrome for human lysine deficiency (16). On the other hand, such treated casein may be toxic for other reasons (26, 145).

The dry heating of proteins may cause an apparent loss of lysine or even histidine, inasmuch as lysine and, to a greater extent, lysine plus histidine additions, after heating restores most of the lost protein nutritive value (161). The nutritive quality of cereal proteins was found to be noticeably impaired with respect to digestibility and biological value by the steam explosion process, with no analytical loss of any of twelve amino acids (266). The lysine in human globin seems to be somewhat less available, nutritionally, when this protein is dried in mild heat (117). Heating casein reduces the effect of the specific enzyme, lysine decarboxylase, on enzymic digests of casein (132). Hot alcohol- or heat-treated liver proteins suffer nutritive damage which may be largely corrected by subsequent acid hydrolysis (followed by restoration of tryptophan destroyed in the hydrolysis (330)). It was first suggested that the damage caused by heat or hot alcohol is stereochemical in nature (72, 330). Later it was shown that there is an apparent loss of free amino nitrogen, but not of total nitrogen, from alcohol-treated proteins; apparently the ϵ -amino groups of lysine are linked to other free groups, probably acidic (as in glutamic acid and tyrosine). These new linkages are difficult to hydrolyze enzymatically but can be hydrolyzed chemically, thus restoring the lysine (169, 72, 68, 285).

Most of the heat damage to the proteins in a cake mix is correctable by adding lysine to the baked, heated or toasted cake (71). Overheated soybean meal protein shows an induced deficiency of

lysine (99, 294, 255, 143, 348). Attention has been directed to the probability that in such complex mixtures some lysine may be lost by reaction with non-proteins, such as carbohydrates (71). Lysine is destroyed by heating with glucose (348). Furthermore, the loss of lysine from proteins admixed naturally with carbohydrates and fats does not seem to be reversed by acid hydrolysis of the feed-stuff (294, 348). This fact suggests a second type of loss of lysine, probably permanent, and involving reactions with non-protein substances.

Tryptophan

There seems to be unanimous agreement that tryptophan or an active substitute is indispensable for growth and maintenance of nitrogen balance in all species in which studies have been made. Both the D- and L-forms promote growth of the rat (70, 127, 55). Rats are also able to use acetyl-L-tryptophan (127, 55), indolepyruvic acid (60, 196), indoleacetic acid (52) and N-methyl tryptophan (89, 151) but not acetyl-D-tryptophan (126, 55) or indolepropionic acid (60). Mice show somewhat less rapid growth when fed the DL-racemic mixture in place of L-tryptophan (358). On the basis of efficiency of feed conversion, white Leghorn chicks do not appear to utilize the D-component of DL-tryptophan (156, 371). However, in comparative tests, a colored variety of chicks was able to utilize up to approximately one-half the D-isomer fed as DL-tryptophan (371). There is no explanation as yet for the different results with the two varieties of chicks.

Adult rats may be kept in positive nitrogen balance on a diet containing as little as 0.2% L-tryptophan (108). Dogs maintain nitrogen balance as well with DL- as with L-tryptophan (244). On a severely tryptophan-deficient diet, human adults enter a period of negative nitrogen balance after a few days, and return to nitrogen equilibrium within a week after tryptophan is restored to the diet (188). Negative nitrogen balance results during the feeding of a protein hydrolysate deficient in tryptophan (112, 47a). Nitrogen equilibrium is maintained in human subjects on as little as 0.2 gram L-tryptophan per day in a total of only 6.7 grams of a mixture of eight amino acids per day. Giving only 0.1 gram of L- or 0.15 gram DL-tryptophan per day results in a negative nitrogen balance (303). A 70 kg. man requires from 0.21 to 0.42 gram of tryptophan per day to reestablish normal tryptophan excretion (190). The most recent estimates based on satisfactory nitrogen balance are that an average human male requires only 0.20 to 0.25 gram of L-tryptophan daily (137, 47a, 304a), or about 3 mg per kg.

of body weight. In contrast, the human infant requires at least 40 mg per kg. of body weight daily (6). It is obvious that human infant requirements cannot safely be interpolated from adult requirements.

The inclusion of the D-isomer of tryptophan in the human diet leads to the appearance in the urine of comparatively large quantities of a substance giving color reactions for tryptophan (9). Strangely, feeding acetyl-DL-tryptophan does not give rise to these color reactions in human urine, and it is suggested that the acetylation improves the utilization of D-tryptophan in man (10). The larger part of intravenously administered acetyl-DL-tryptophan was found unchanged in the human urine within 6 hours of the injection (241). It appears, therefore, that the method of administration has a great influence on the efficiency with which acetylated tryptophan is utilized. It has been reported that the human infant will show equally satisfactory gains and nitrogen retention when required to utilize L-tryptophan, acetyl-L-tryptophan and acetyl-DL-tryptophan, but not in the case of DL-tryptophan (6). Most of the D-component is found to be excreted unchanged when DL-tryptophan is fed to infants (291). Neither D- nor acetyl D-tryptophan have any detectable influence on nitrogen balance in adult humans (47a, 304a).

It has been estimated on the basis of a colorimetric analysis for tryptophan that the normal human adult excretes in the urine about 3.3 mg. of tryptophan per kilo of body weight daily (8). However, a microbiological determination of tryptophan in human urine yielded only about one-tenth of the estimate (327).

Certain symptoms have been described as accompaniments of tryptophan deficiency. Rats develop acute and chronic cataracts, globular degeneration of the lens fiber, and proliferation of the epithelium (359, 5). The ocular lesions are apparently due to a direct effect on the cornea (4). The rats also show loss of weight, hunchback, unkempt appearance, alopecia, greasy hair, and nervousness. To these conditions have been added defective teeth, testicular atrophy, hypoproteinemia, hypochromic anemia, and failure of gestation (22). It has been reported that rats completely deprived of tryptophan for a few days become permanently sterile (213). However, a careful re-investigation has shown that the reported sterility could not have been caused by a tryptophan deficiency (58). In humans, no tryptophan deficiency symptoms other than loss of nitrogen have yet been induced experimentally (189).

Tryptophan has been linked to nicotinic acid (niacin), in the

prevention of pellagra-like-symptoms. Corn has long been known as an etiological factor in the diets of pellagrins. Corn proteins are notoriously poor sources of tryptophan; and corn grain is one of the lowest in niacin content. Corn added to a synthetic diet for dogs increases their niacin requirements (229). On a low protein, high-corn diet rats show poor growth, which is improved by adding 1 mg. niacin per 100 grams diet. Strangely enough, 50 mg. of L- or DL-tryptophan has the same effect (228). Very similar results have been obtained with the guinea pig (91). The interchangeability of niacin and tryptophan in such diets has been confirmed in paired-feeding experiments which minimize the effect of changed appetite (340).

The addition of 0.5 gram L-tryptophan to the high-corn diet caused an immediate increase in the urinary excretion of niacin (337). The total niacin found in the rat (plus excreta) was greatly augmented by added dietary tryptophan (227). Adding tryptophan to a rat diet containing an adequate protein source further stimulated the excretion of niacin and its derivatives (311, 337, 53). The amino acid imbalance in proteins such as zein and gelatin, as well as their low tryptophan contents, is evidently partly the reason for their pellagrigenic activities in the diet of the rat (226, 53) and the chick (84, 164). Human infants and adults responded with an increased output of niacin and its derivatives when given generous amounts of DL-tryptophane (291, 317).

An amino acid imbalance produced by adding tyrosine or phenylalanine to a 10% casein diet for rats caused growth retardation and lesions which were appreciably alleviated when niacin or tryptophan was added (279).

It has been suggested that corn contains a substance which displaces niacin from its normal metabolic paths, in this way bringing about the apparent pellagrigenic properties of this grain (385). The interference with normal metabolites by their structural analogs is a well proven phenomenon. For example, 3-acetyl pyridine given to mice causes a disease closely resembling human pellagra or canine blacktongue (385). The effects of 3-acetyl pyridine can be overcome by adding niacin or tryptophan to the diet (387). In support of this viewpoint, extraction and concentration of a pellagrigenic agent from corn has been reported (388). The agent appeared to be indoleacetic acid (221). However, this compound was previously found not to be inhibitory to niacin (226) and, the inactivity of indoleacetic acid in the tryptophan-niacin relation in rats has been confirmed (312).

Tryptophan-deficient proteins in the diet of chickens will in-

crease the niacin requirement of this animal for growth, egg production and hatchability (84). In the pig, niacin deficiency cannot be demonstrated except on low-protein diets, and there appears to be a close nutritional relationship between proteins, tryptophan and niacin synthesis (374, 237). This may well involve tryptophan. While the explanation of the interaction between tryptophan and niacin is still incomplete, the experimental facts show that tryptophan and niacin are interchangeable, within limits.

In contrast to niacin deficiency, the effects of pyridoxine deficiency are aggravated by high-protein diets or by added tryptophan (262, 47). It appears that pyridoxine is involved in the tryptophan-niacin relationship (53). High-protein diets may increase pyridoxine requirement (328, 333, 95). This vitamin is intimately concerned in protein and amino acid metabolism (241). (See review 349a.)

Methionine and cystine

The sulfur-containing amino acid, methionine, is a necessary component of all animal diets. In addition, it helps to meet the physiological requirement for cystine by furnishing the sulphur atom for the biological synthesis of cystine (352, 351, 310). Homocystine is capable of replacing methionine in the diet of the rat for growth purposes only when a significant amount of dietary choline is present (122). This is also true with the chick (218). Evidence of the transfer of methyl groups from choline to homocystine, thus forming methionine, was obtained by feeding homocystine together with choline in which the methyl groups were labeled with deuterium. A rapid and continuous transfer of methyl groups takes place from choline to form tissue methionine irrespective of the intake of the sulfur containing amino acids (336).

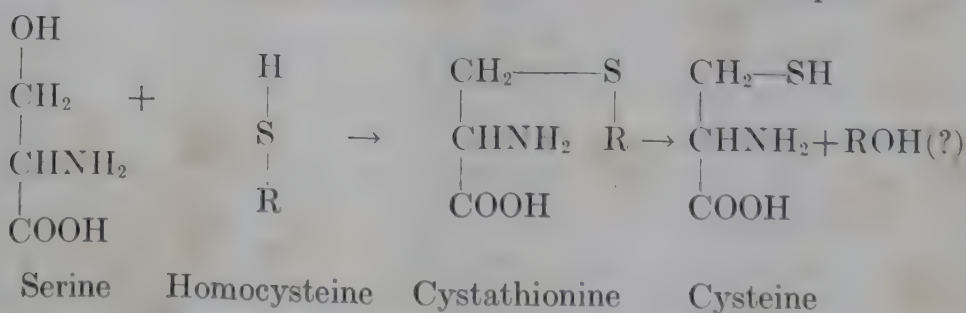
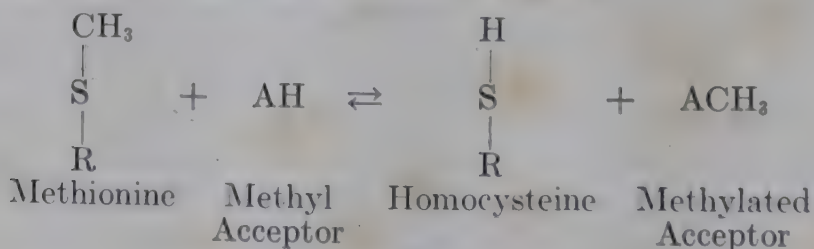
Rats are at times able to achieve slow growth on methionine-free, choline-free diets containing homocystine.* The condition of the rats as determined by the pre-experimental diet is evidently a factor affecting somewhat the ability of the rat to grow on such a diet. A non-absorbable, bactericidal substance, such as succinyl-sulfathiazole, in the diet appears to destroy this ability; hence, choline synthesized by micro-organisms in the intestines may have helped to convert the homocystine to methionine (54).

Chicks have been shown to utilize homocystine for slow growth on methionine-low, choline-free diets (34, 155), although severely

* The rate of growth under these conditions can be greatly increased by ample amounts of folic acid and of vitamin B₁₂ (344a).

depleted of choline in advance of the experiments. The chick has much less ability than the rat to synthesize choline. It is well known that choline-depleted animals still retain a normal content of bound choline in the tissues. Also, tissue choline engages in methyl group exchange with methionine (102, 123). The following comment seems to remain appropriate: "One must also recognize the possibility that tissue-bound choline may be able to promote methylation processes" (30). It seems entirely possible that some of the above-mentioned growth could be due to methylation of homocystine by tissue-bound choline. This activity would, perhaps, be especially effective in diets containing an adequate level of cystine. In diets low in both choline and cystine, homocystine may still be utilized as a precursor of cystine by both the rat (66) and the chick (155).

A compound which can be regarded as a condensation product of serine and homocystine is cystathionine ($\text{HOOC} \cdot \text{CHNH}_2 \text{CH}_2 \cdot \text{CH}_2\text{S} \cdot \text{CH}_2\text{CHNH}_2 \cdot \text{COOH}$). This thioether is capable of replacing cystine, but not methionine, in the diet. It is apparent that cystathionine splits to give cysteine, but no appreciable homocystine, and that it may be an intermediate stage in the *in vivo* synthesis of cystine from methionine and serine (121). This hypothesis was strengthened by the preparation of methionine labeled with S^{34} , and with C^{13} in the β and γ positions, and then feeding this doubly-labeled methionine to rats. Isotopic analysis of the rat tissue cystine showed that approximately 80% of its sulfur, but none of its carbon, had been derived from methionine (125).



A = dimethylamino ethanol, for example; or guanidoacetic acid. The reaction is not reversible with the latter.

Four cystathionines are possible and have been synthesized (43). According to the nomenclature proposed (43) and the current system of indicating configuration, the compounds are named as follows:

	3-carbon part	4-carbon part
L-cystathionine	L	L
D-cystathionine	D	D
L-allocystathionine	L	D
D-allocystathionine	D	L

Of these compounds, the second and fourth do not support rat growth when added to a cystine-free, methionine-restricted diet; hence are not appreciably converted to either amino acid. L-allocystathionine will rectify a diet deficient only in methionine, but choline is necessary for its activity. Thus, L-allocystathionine splits to yield homocysteine, in contrast to L-cystathionine which yields cysteine (43). If the scission of L-cystathionine is in the nature of a hydrolysis or a reduction, detectable amounts of either α -amino- γ -hydroxybutyric acid or of α -aminobutyric acid should be formed in metabolism. The latter has been obtained from urine after methionine feeding (114). The former (homoserine) has been shown to act as an intermediate in the synthesis of both threonine and methionine in *Neurospora* (353). DL-Homoserine will not, however, replace methionine in the diet of the rat, even in the presence of cystine and choline (44).

Despite the fact that only the L-isomer is useful in the synthesis of cystine by liver slices, racemic-methionine is fully as effective for growth purposes as the natural form when added to rat diets very low in both methionine and cystine (302, 198). Either isomer of methionine is effective for the growing mouse (51). Balance experiments with dogs and humans indicated no difference between L- and DL-methionine (344, 304a). In the diet of the chick, L- and DL-methionine are equivalent for growth irrespective of large variations in the dietary cystine and choline content (155, 158). In man, D-methionine is utilized as readily as the natural form (2) and the total sulfur-containing amino acid requirement can be met by methionine (306, 12). The urinary excretion of methionine by normal humans is surprisingly high (2, 355), amounting to approximately 4.5 mg. per kg. of body weight per day. The excretion seems to be relatively independent of gross protein intake, but increases when the free amino acid is given, and decreases during a fast, or ingestion of a high fat diet (355).

A cystinuric subject will excrete cystine when given methionine,

cysteine, glutathione, alanine, glycine or glutamic acid, but not when given cystine (178, 179). In the face of such results, the excretion of the extra cystine can hardly be attributed to anything more than a general stimulation of protein metabolism (78). The apparently negative results obtained when cystine is fed are peculiar, but it may be that this large and comparatively insoluble amino acid is both slowly absorbed and poorly utilized in cystinurics, *i.e.*, they do not readily reduce it to cysteine either before or after absorption.

DL-cystine, when given to normal humans, causes a cystinuria which is not observed on administering a similar quantity of L-cystine. The urinary cystine is in the D-form. However, approximately 75% of the D-cystine ingested has not been accounted for and may, therefore, have been metabolized along normal paths. Thus the urinary loss of D-cystine which occurs on feeding some protein hydrolysates would not seriously affect the nutritive value of such preparations (3).

Cystine stimulates growth only when methionine is furnished in amounts insufficient to meet the total sulfur-containing amino acid requirements (33, 225a, 381). Cystine cannot fully compensate for a deficiency of methionine below the critical minimum requirement, but unless the deficiency is so extreme as to render the animals moribund, cystine will always exert some methionine-sparing action.

Lanthionine is a thioether derived from cystine by the removal of one S-atom during the treatment of proteins with alkalies that is isolated in the meso and racemic form. The internally compensated mesolanthionine cannot serve in lieu of cystine in the diet (146). This implies that it is either not split at all, or only to D-cysteine, which is not utilized by the rat for growth. However, the L- or DL-forms of lanthionine can be utilized in a cystine-deficient diet (202), indicating that L-cystine may be derived from them.

Methionine has one function in common with choline, that of a contributor to the pool of "labile methyl groups." Complete absence of choline in the diet of the rat may be compensated by sufficient methionine because the synthesis of the remaining moiety of choline proceeds with facility (122, 345).

The chick is a species in which total choline synthesis is, at best, a very limited process (27, 204). In this species, dietary methionine is able to replace choline only to the extent of their common function, *i.e.*, methylation (27, 30). If dimethylaminoethanol is given the chick, the latter is then able to form the choline needed to pre-

vent choline-deficiency perosis and restore growth (208, 209). It is interesting that the "choline-less" mutant of *Neurospora crassa*, like the chick, seems unable to use methionine or betaine in synthesizing choline, unless given dimethylaminoethanol (207, 192). The turkey, also, cannot utilize methionine as a complete substitute for choline (205).

Methionine is the only identified direct carrier of methyl groups for the conversion of guanidoacetic acid to creatine by liver slices (77, 123). While a single or combined deficiency of arginine and glycine (precursors of guanidoacetic acid) will lead to a significant lowering of chick tissue creatine content, a lack of methionine (and also of choline) causes no more than a slight effect (39). The creatine content of the gastrocnemius muscle of young rats is not lowered during a dietary deficiency of methionine and choline (297), nor is the relative excretion of creatine decreased (354). It seems strange that lack of either of two of the amino acid precursors of creatine should be serious with respect to creatine formation (in the chick) while lack of the third precursor, methionine, is not. The suggestion has been made that creatine formation in the rat takes precedence over other demands on the labile methyl supply (354). The growth-depressing action of an excessive methionine intake in the chick may be alleviated by the simultaneous ingestion of guanidoacetic acid; hence it would seem that the methionine was readily destroyed by demethylation induced by the guanidoacetic acid (257). When guanidoacetic acid is added to a glycine- and arginine-deficient chick diet, the chick responds with an increase in growth, and creatine accumulates to a large excess over normal in the chick liver, the creatine being formed faster than it can be carried away (39). Evidently, in the chick, the methylation phase of creatine formation is a fast reaction in comparison to the prior steps in which guanidoacetic acid is formed from glycine and arginine.

Rat diets containing 18% casein as the sole source of protein are slightly deficient in sulfur-containing amino acids and may be improved for growth by adding cystine (271, 290). There is barely sufficient methionine in such diets when the choline and cystine levels are made adequate. At this and lower casein levels, a choline deficiency induces hemorrhagic degeneration of the kidneys of young rats. The choline deficiency syndrome is overcome by raising the casein level to at least 30%, thus providing sufficient extra methionine for choline synthesis (271). That so much extra methionine must be provided is due to the fact that three molecules of

methionine are required to furnish the methyl groups for one molecule of choline, a fact sometimes overlooked.

While it has been suggested, on the basis of physiological data, that methionine which yields up its methyl groups cannot at the same time serve as a precursor of cystine (271), such evidence does not harmonize with the facts that methyl groups, homocysteine and methionine are reversibly related, and that the homocysteine is a known precursor for cysteine. A critical test of this question has not yet been made, but could probably be carried out by labeling both the methyl group and the S-atom of methionine, and then testing for these groups in tissue choline, creatine and cystine after feeding.

Choline deficiency in adult rats is produced only on diets which are low in both methionine and choline. It causes fatty enlargement of the liver, terminating in cirrhosis. On low protein diets, rats, in time, may develop a massive acute liver necrosis, resembling yellow atrophy of the liver in man. Twenty mg. of methionine per rat per day is completely protective (148).

A direct application of the experimentally demonstrated interrelations between the sulfur-containing amino acids, choline and liver fat has been made in human cases of fatty degeneration, enlargement, and cirrhosis of the liver (287). The combination of cystine and choline has usually had a favorable effect in the treatment of cirrhosis when tissue damage was not too far advanced. Methionine has been found to be an effective agent, also, in the treatment of liver cirrhosis. High protein diets (high sulfur-containing amino acid intake) plus choline are helpful. References to clinical reports may be found in recent reviews (280, 97, 163, 206).

Toxic hepatitis, caused by exposure to trinitrotoluene has been treated favorably by high protein diet, and more specifically by cystine plus choline, or by methionine (130). Methionine has been found useful in the treatment of liver injury due to mapharsen (149) (3-amino-4-hydroxyphenylarsinoxide hydrochloride). There are several reports of the beneficial effect of methionine, choline plus cystine, or high protein intake in promoting recovery from liver damage caused by a variety of toxic agents. Conversely, low protein diets render an animal more susceptible to liver damage by such toxic agents (281). It is quite possible that the favorable action of methionine is multiple, *i.e.*, it serves as a precursor of choline (lipotropic factor), and of cystine (detoxifying factor) and of both sulfur-containing amino acids for repair of diseased tissue.

Methionine, homocysteine or cystine added to a diet containing

an inadequate amount of casein caused a significantly faster restoration of depleted liver protein of the starved rat. Neither choline nor alanine was similarly effective, indicating that the effect of methionine was not due merely to methylation capacity or to the provision of amino nitrogen (170). Methionine, apparently, is the most effective single amino acid for increasing the N retention of rats and dogs on low-protein diets (85, 263, 24). This effect, however, has not been demonstrable in humans (111, 201).

Raw soybean protein exhibits a peculiar defect which is largely overcome when methionine, but not cystine, is added, either as such or in supplementary proteins (40, 173, 32). Raw soybean meal serves quite well in a basal methionine deficient diet for methionine bio-assay (158). Heating of the soybean meal can be conducted in such a way as to overcome most of the protein defect (99, 294, 143, 255).

Certain compounds structurally related to methionine have not been able to replace it in the diet. These include the ethyl analog, ethionine (129); the oxygen isostere, methoxynine (298); and thienylalanine (126). The latter also was unable to replace phenylalanine.

Phenylalanine and tyrosine

Phenylalanine is required in the diets of growing rats (302), mice (51), chicks (31), adult dogs (308), and adult humans (307). It is also a precursor of tyrosine and, if given in sufficient quantity, can eliminate the need for dietary tyrosine (302, 31, 51, 309, 306, 152). At adequate intakes of phenylalanine, tyrosine has a "sparing action" and will promote faster growth of either the rat or the chick; but if the deficiency of phenylalanine is extreme, the presence of tyrosine can accomplish little or nothing (31, 383, 152). Direct *in vivo* conversion of deuterium-labeled phenylalanine into tyrosine has been demonstrated even in the presence of large amounts of tyrosine in the diet (367).

The phenylalanine and tyrosine contents of chick tissue proteins remain constant regardless of variations of these amino acids in the diet, and of variations in growth rate (153). Similar results have been obtained with rats in which tyrosine, cystine and tryptophan were studied (236).

D-phenylalanine seems to be almost equally as potent as the natural form for the rat (302, 309), mouse (51), and chick (152); however, there is considerable variation in its utilization by man (2, 21, 304a). Very soon (7 hours) after the oral administration of DL-

tyrosine, humans excrete in the urine a marked excess of tyrosine and organic acids which are apparently sufficient to account for nearly all the D component fed. Since none of the acids resemble known products of incomplete L-tyrosine metabolism, D-tyrosine is, presumably, unavailable for normal purposes in man (20).

In *Oligophrenia phenylpyruvica* there is a failure in the metabolism of phenylalanine which results in the appearance in the urine of phenylpyruvic acid (138). The affected subjects appear to be unable to oxidize this acid at a normal rate (200). Not all the ingested phenylalanine may be accounted for as phenylpyruvic acid and the other paths of phenylalanine metabolism are apparently not impaired. Feeding D-phenylalanine results in the excretion of more phenylpyruvic acid than when the L-form is fed, which again suggests that in man the D-form is less readily metabolized along the other paths (200).

A phenylalanine deficiency in the chick leads to a peculiar deformity of the tongue which is curable by restoring the phenylalanine intake. The same symptom has been noted with deficiencies of leucine and isoleucine, but not with any other amino acid or protein deficiencies (153).

Vitamin C is evidently concerned in the metabolism of phenylalanine and tyrosine. In vitamin C deficiency, *p*-hydroxyphenylpyruvic acid, homogentisic acid and other products of incomplete metabolism are excreted in the urine (329, 238, 233, 50). These products disappear when vitamin C is given. In the chick, the feeding of increasing amounts of tyrosine has been reported to cause a decreasing level of ascorbic acid in the liver (182). The oxidation of tyrosine by the liver tissue of rats varies with the quantity of pteroylglutamic acid (folic acid) present (300). Added tyrosine or phenylalanine increases the niacin (or tryptophan) requirement of rats (279).

Leucine

All lists issued on the amino acids which must be provided in the diets of experimental animals have included leucine, except for one report (84). No symptoms of the deficiency other than the quite general effects of lack of growth, loss of weight, negative nitrogen balance and impaired appetite have been reported, with the exception of a tongue deformity in the chick (153).

Only the L-leucine is used by the rat (302) and mouse (51) for growth; however, the chick appears able to employ the racemic form so efficiently that most of the D-leucine must be utilized by this

species (160). The rat is able to aminate α -keto- γ -methylvaleric acid, since it will grow well when this source of the carbon chain of leucine is given in a leucine-deficient diet (302).

Ability of the adult rat to invert D-leucine to some extent may be shown by feeding the isomer labeled with N^{15} and with D attached to the carbon chain. While the N^{15} is widely scattered among the tissue proteins and amino acids, the deuterium-labeled carbon chain appears in the L-leucine of the tissue proteins (292).

Isoleucine

Isoleucine, differing only in the position of a methyl group as compared to leucine, is similarly indispensable in the diet of all species tested. The rat (302), mouse (51) and chick (160) do not utilize D-isoleucine for growth. Rats deprived of isoleucine will grow when given α -keto- β -methylvaleric acid; hence, this compound is undoubtedly aminated to isoleucine.

A deficiency of isoleucine is a limiting factor in the nutritive value of beef blood red cell proteins (157, 284), and of human globin (117). Beef hemoglobin protein has been used in a basal diet for estimating the human infant requirement for isoleucine. This requirement was set at 90 mg. of L-isoleucine per kg. per day (14).

Valine

In every instance, valine has been found necessary in the diet for growth, maintenance, or nitrogen balance. The D-antipode is not utilized by the rat (302), mouse (51), or chick (160). L-hydroxyisovaleric acid may be substituted for valine in the diet (301). Young rats deprived of valine exhibit the usual symptoms of complete nutritive failure. In addition, they become extremely sensitive to touch and develop a marked incoordination of movement (302). The latter symptom is not observed in chicks on a completely valine-deficient diet, but there is a rapid decline, very soon terminated by death (31). Valine deficiency in man results in an immediate negative N balance (306).

Threonine

Threonine, first demonstrated to be a dietary requirement for the rat (302), is probably necessary for all species at all times. This amino acid has two asymmetric C atoms, hence four stereoisomeric forms. Only one of these, L-threonine, is known to be physiologically active (368). Studies on chick, dog and human requirements have not shown whether the D component of racemic threonine is uti-

lized. Lack of threonine has been reported to cause an edematous condition in the mouse (51). The conditions which are usually employed for complete hydrolysis of proteins do not seem to cause a loss of threonine (75).

Glycine

To meet normal needs the rat does not require an exogenous supply of glycine. The rat couples benzoic acid with glycine to form hippuric acid (296). When much benzoic acid is given without glycine the growth rate is impaired, but it may be restored by giving glycine in addition to the benzoic acid (162). It seems evident that under such abnormal conditions the rat cannot meet both the growth and detoxifying requirements for glycine by synthesis alone. Glycine is regarded as necessary in the regeneration of plasma proteins in the dog (252).

In the chick, a dietary supply of glycine is requisite to optimal growth (25, 31, 38, 41). Limited synthesis of glycine undoubtedly takes place (25, 31) even in the embryonic stage (289), but is not sufficient to meet the demands of early rapid growth. A glycine-deficient chick diet is improved for growth by adding acetate (35). Dietary glycine deficiency in the chick is accompanied by a lowered muscle creatine content and a profound weakness. Feeding creatine, creatinine or glycocyamine, for all of which glycine is a precursor, restores growth, strength and muscle creatine content (35, 30).

A comparatively higher demand for glycine in the case of a more rapidly feathering variety of chickens has been explained as due to a high content of glycine in feather protein (175). Young turkeys do not exhibit evidence of glycine deficiency as readily as the chick (204), but recent reports indicate a positive requirement (210, 225).

While the chick exhibits maximal growth at 1 to 1.5% glycine in the diet (38), a slightly higher level is apparently harmful (41, 38). Glycine, in large doses, is also toxic to hens (289). Gelatin, a protein which contains 25% glycine, is not well tolerated by dogs at continued high levels of intake (299). This protein, even when supplemented with all its lacking indispensable amino acids, still fails to support good growth (181). Glycine, or proline (which is also plentiful in gelatin) when given at 5% or more of the diet, is found to be inhibitory to growth (181, 216). It seems likely, therefore, that the toxic effects of glycine begin to make themselves felt at levels not far above the optimal intake. Despite the fact that this amino

acid is the simplest and cheapest, its use in amino acid therapy must not be indiscriminate.

Chicks on a niacin-low diet are precipitated into a pellagric condition by feeding them extra glycine, arginine, alanine, gelatin, or zein. Adding niacin or tryptophan overcomes the syndrome and increases the tolerance of the chick for abnormally high intakes of amino acids (84, 164). The amino acids mentioned are especially prevalent in cartilaginous tissues, and it may be more than a coincidence that the pellagric syndrome in the chick includes a derangement of cartilaginous structures of the leg (perosis).

The inclusion of some glycine in amino acid mixtures for parenteral administration has a beneficial effect in increasing the tolerance for the D-amino acids given intravenously in racemic mixtures (194), and for glutamic acid (251). The addition of glycine to the diet in some cases seems to increase the appetite and body weight of underweight individuals and of patients with muscular dystrophy and atrophy (360).

The fully nitrogen-methylated glycine (glycine betaine, more commonly referred to as betaine) is a substance of quite general occurrence in foods. Consequently, it is of interest, practically as well as theoretically, for its relation to both glycine and choline. Betaine labeled with N^{15} is converted successively to glycine, ethanolamine and choline in the rat, but is not reduced directly to choline (345, 128). From glycine on, the metabolism is the same as that of serine (332, 345). In the chick, however, the difficulty in the synthesis of glycine apparently extends also to the utilization of betaine, which does not replace glycine in the diet for growth purposes (35). Betaine will function in the chick as a source of methyl groups, *i.e.*, it can replace choline for the methylation of homocystine (30). For methylating purposes, betaine has a "sparing action" on choline in the chick (257, 17). The utilization of dietary betaine as an effective methylating substance in the rat is well known (268, 128). Although non-methylating, arsenocholine can provide all other known functions of choline for the chick. A combination of arsenocholine and betaine is an effective complete substitute for choline in the chick (30).

Under conditions of severe choline deficiency, betaine alone is of little or no value in the chick since the absence of other functions of choline is a severe handicap. It is evident, therefore, that in the chick, betaine is neither demethylated to glycine nor is the carboxyl group of betaine appreciably reduced to an alcohol group for the formation of choline.

Serine

The nutritive rôle and the function of serine in metabolism has already received mention in preceding sections on amino acids. Serine, together with methionine, is a precursor for cystine (121, 346); it is readily converted to glycine (332), ethanolamine and ultimately choline in the rat (345). Serine is dispensable in the diet; hence, since it is a necessary physiological substance, it must be readily synthesized (302, 31). Isotopic experiments have demonstrated the formation in the rat of serine from glycine (315, 374a), and possibly from glutamic acid (332).

A comparison of the relative toxicity of DL- and L-serine in rats reveals deleterious effects from the DL- but not from the L-form. Loss of weight, renal necrosis, proteinuria, excretion of reducing substances and of serine itself, follow the administration of DL-serine by stomach tube. It appears that the D-isomer is chiefly responsible for the symptoms noted (137).

Alanine

Elimination of alanine from the diets of rats (302) and chicks (31) seems to create no difficulties for these animals. This amino acid is evidently readily synthesized by all species. In direct contrast to DL-serine, DL-alanine in the diet leads to no noticeable toxic effects and no abnormal substances in the urine (137).

As a general source of amino acid nitrogen, alanine has a minor but useful place in amino acid mixtures for experimental diets or for oral and parenteral administration. The possibility that alanine might be a biological precursor of pyridoxine is indicated by the fact that alanine will help to replace this vitamin for the growth of certain microorganisms (339). An isomer, β -alanine is a component of the vitamin, pantothenic acid.

Glutamic acid

Glutamic acid is seemingly readily synthesized by all animals, except the young chick, which profits from a dietary supply of glutamic acid, and is unable to synthesize it at a rate quite sufficient for all the needs of early life (19). Additions of glutamic acid stimulates the growth of rats on a ration containing only the ten essential amino acids (307a). A close interrelation between glutamic acid, arginine and the prolines in the rat has been reviewed and further investigated by tracer studies (347). A re-examination of the

dietary status of glutamic acid for the rat has shown that diets devoid of glutamic acid, arginine, proline and hydroxy-proline are significantly improved for growth by the addition of either proline or glutamic acid, although neither is as effective as arginine (383). This study furnishes additional evidence for the biological inter-conversion of glutamic acid, arginine and proline.

Glutamic acid in intravenously administered amino acid mixtures or protein hydrolysates has been identified as one cause of the nausea and vomiting often observed (251, 361). The low tolerance to an intravenous casein hydrolysate is due primarily to its high (over 20%) content of glutamic acid (251, 361), and perhaps to its very low content of glycine, since glycine increases the tolerance to glutamic acid (251).

Glutamic acid is one of the three amino acids in the widely occurring peptide, glutathione. More recently, glutamic acid has been identified as a component of the folic acid vitamins, which contain 1, 3, or more molecules of glutamic acid coupled to pterin and para-aminobenzoic acid. Serylglycylglutamic acid possesses some of the activity of streptogenin, a growth factor for certain micro-organisms (389). Of unusual interest is that glutamic acid administration is said to improve intellectual performance, especially of subnormal persons (282).

Aspartic acid

A dietary requirement for aspartic acid has not been determined for any species (302, 31, 305). This amino acid, like glutamic acid, may not be very well tolerated by the intravenous route, and has been reported as another cause of vomiting (361, 245).

Proline and hydroxyproline

While seemingly not required in otherwise complete diets for the rats (302, 384), proline is synthesized adequately by the chick only after a lag of a few days, probably to allow the development of a synthetic process (31).

Since a deficiency of either proline or glutamic acid is at least a temporary handicap to growth of the chick, it may well be that these amino acids, and possibly ornithine, are interrelated in the chick as well as in the rat. From a growth response standpoint, hydroxyproline appears ineffective in these interrelations, hence its conversion must be comparatively slow (384).

Utilization of D-isomers and α -keto, α -hydroxy and α -N-methyl analogs of amino acids

Theories which have been proposed to account for the fact that D-isomers of certain amino acids are utilized do not explain why D-isomers of other amino acids are apparently not utilized, or why there are species differences in their utilization (Table I). For example, there is no doubt that amino acids may be deaminated and

TABLE I
UTILIZATION OF D AMINO ACIDS IN VARIOUS SPECIES

	Chicken	Mouse	Rat	Human
Methionine	+ (155)*†	+ (51)	+ (198, 302)	+ (2, 304a)
Cystine			— (124)	+ (3)
Phenylalanine	+ (152)	+ (51)	+ (309, 302)	+ (21, 304a) — (2)
Tyrosine				— (20)
Tryptophan	— (156) + (371)	+ (358)	+ (55, 127, 57)	— (9, 6, 291, 47a, 304a)
Histidine		+ (358)	+ (103)	— (11)
Lysine		— (358)	— (56, 276, 293)	— (304a)
Isoleucine	— (160)	— (51)	— (302)	— (304a)
Leucine	+ (160)	— (51)	+ (292) — (302)	— (304a)
Valine	— (160)	— (51)	— (302)	— (304a)
Threonine	— (154a)		— (368)	— (304a)
Arginine			— (19)	+ (19)

* References to literature in parentheses.

† Where indicated as positive the degree of utilization of the D isomer is often comparatively poor, and where indicated as negative some slight degree of utilization is not necessarily disproven.

converted to α -keto or α -hydroxy analogs. Many of these analogs will replace the corresponding amino acids in the diet and undoubtedly may be reaminated (Table II). Thus, for example, D-methionine may be rapidly deaminated *in vivo*, and the L-form resulting from reamination then continuously removed by biological utilization. Such an inversion process must compete with others which result in the destruction or excretion of the D-form. It is conceivable that some metabolites of a D-amino acid may be identical with metabolites of the L-form, and possibly exert a "sparing action" on the L-amino acid.

The results indicated in Tables I and II are by no means absolute. Their real significance may apply more truly to rates of conversion.

2. Quantitative Requirements for the Amino Acids

It is obvious that when only nine or ten amino acids are fed, the animal must use a goodly portion of the fed amino acids in the synthesis of others for the building of tissue proteins. Consequently, requirement data based upon such a dietary regime are very likely

TABLE II

UTILIZATION OF ALPHA-KETO, ALPHA-HYDROXY, AND ALPHA-N-METHYL ANALOGS OF AMINO ACIDS FOR GROWTH

	Alpha-Keto Acid	Alpha-Hydroxy Acid	Alpha-N-Methyl Acid
Methionine	+ (90)*	+ (1, 69)	+ (288)
Cystine		— (369)	+ L — D (215)
Phenylalanine	+ (301)	+ (301)	
Tryptophan	+ (196, 60)	+ (195, 52)	+ (151, 89)
Histidine	+ (171)	+ (107, 171)	+ (136)
Lysine		— (256)	— (150)
Isoleucine	+ (301)	+ (301)	
Leucine	+ (301)	+ (301)	
Valine		+ (301)	
Arginine		+ (76)†	

* References to literature in parentheses.

† Very poor utilization.

to be much higher than minimal requirements determined with a complete assortment of amino acids. The quantitative needs of the rat and chick which have been estimated on the latter basis are listed in Table III.

The estimated cystine requirement (381) of the rat (0.1%) at a minimal methionine level (0.5%) may be too low. These levels are furnished by 18% casein in the diet and this protein is known to be supplemented favorably by added cystine (271, 290). The cystine requirement of the chick at minimal methionine intake is approximately 0.4% of the diet (155).

Approximately twice the indicated amount of phenylalanine is needed by the rat or chick if no tyrosine is present in the diet. The maximum tyrosine requirement is 0.6 to 0.8% for the chick (160)

and 0.5% for the rat (278), when the minimal adequate levels of phenylalanine are provided.

Chicks will grow, although very poorly, on only the amino acids listed in Table III (174). Unfortunately, such a study does not provide data on the true minimal requirements. It has been emphasized that the data for the rat must still be regarded as provisional (309). In many cases, the amino acid requirement data have

TABLE III
MINIMUM PERCENTAGE OF EACH AMINO ACID NECESSARY IN THE
DIET TO SUPPORT GOOD GROWTH WHEN ALL OTHER AMINO
ACIDS OF NUTRITIVE IMPORTANCE ARE PROVIDED

Amino Acid	Rat	Reference	Chick	References
Arginine	0.2	380	1.0 1.2	220 28, 40a
Histidine	0.4	380	0.15-0.3	28
Lysine	1.0	380	0.9	37, 28
Tryptophan	0.2	380	0.25 0.18	28 372
Methionine	0.6 0.5	380 381	0.5-0.6 0.5	155 28, 29a, 257
Phenylalanine	0.7 0.4	380 383	0.9 0.8	28 152
Leucine	0.8	380	1.4 1.5	28 160
Isoleucine	0.5	380	0.6 0.5	28 160
Valine	0.7	380	0.8 0.7	28 160
Threonine	0.5	380	0.6 0.45	28 154a
Glycine	0.0	302	1.0-1.5	38

been reported on the basis of growth rates which are far below the maximum obtainable. One may suspect, therefore, that some of the growth requirements have been underestimated, and will be revised upwards as the diets are perfected to permit more nearly optimal growth.

The principal criteria for direct assessment of amino acid requirements of man have been nitrogen balance experiments. Indirect estimates of protein and amino acid requirements have been made on the basis of intake and analysis of proteins present in an average "good" diet. Such estimates have been prepared by various

workers and summarized (70). The "optimal" daily intake of amino acids based on diet analysis is compared in Table IV with available data on minimal requirements as found in balance studies.

The first column of Table IV represents the amino acids in 90 to 100 gm. of food protein of good quality (the average American intake). A smaller amount, 70 gm. is suggested as a satisfactory allowance (275). This is equivalent to 1 gm. per kilogram of body

TABLE IV
AMINO ACID REQUIREMENT OF HUMANS
(70 kg. Body Weight)

Amino Acid	Analysis of Optimal Diet (70)	Minimal Content in Certain Proteins at Nitrogen Balance (172)	Requirement for Nitrogen Balance with Basal Diet of Known Deficiency	References for Column 4.
Arginine	g/day 3.5	g/day 1.2	g/day 0	303, 189, 304a
Histidine	2.0	0.5	0	15, 304a
Lysine	5.2	0.8	0.4 to 0.8	304, 304a
Tryptophan	1.1	0.4	0.15 0.20 0.15-0.25	303 13, 47a 304a
Methionine + Cystine	3.8	0.7		
Methionine			1.10	304a
Phenylalanine	4.4	1.4	0.8 to 1.1 1.10	304 304a
Leucine	9.1	1.7	0.50-1.10	304a
Isoleucine	3.3	1.2	0.70	304a
Valine	3.8	1.1	0.80	304a
Threonine	3.5	1.0	0.50	304a

weight for a person of average size. A most careful study of human protein requirements by the balance technique has shown that healthy adults require from 25.7 to 26.5 gm. of protein per day at an average body weight of 70 kg. when meat is included in the diet, and 29.3 to 32.9 gm. when the protein is all of vegetable origin (176). From similar balance requirements and the reported amino acid analyses of four common food protein sources of widely different composition, the minimal quantities of certain amino acids present were calculated (172). While these estimates are not neces-

sarily true minimal requirements, they do approach the few values in the third column of Table IV, which were obtained with basal diets of known deficiencies.

By means of balance experiments on healthy male graduate students, Rose (304a) has determined the amino acids of the diet that are essential for the maintenance of nitrogen balance in the human and has evaluated the minimum required quantity of each amino acid. The subjects were fed a mixture of pure amino acids

TABLE V
MINIMUM AND RECOMMENDED INTAKES FOR NORMAL MAN
WHEN DIET FURNISHES SUFFICIENT NITROGEN FOR
SYNTHESIS OF NON-ESSENTIALS (304a)
(Strictly Tentative Values)

Amino Acid	Minimum Daily Requirement	Recommended Daily Intake	Subjects Tested
	gm.	gm.	no.
L-Tryptophan	0.25	0.5	31 ¹
L-Phenylalanine	1.10	2.2	22
L-Lysine	0.80	1.6	27
L-Threonine	0.50	1.0	19
L-Valine	0.80	1.6	23
L-Methionine	1.10	2.2	13
L-Leucine	1.10	2.2	8
L-Isoleucine	0.70	1.4	8

¹ All of these subjects have been kept in balance on 0.3 gm. or less.

flavored with lemon juice and sugar. The diet was supplemented with wafers made of corn starch, sucrose, centrifuged butter fat, corn oil and vitamins. The nitrogen of the diet over and above that derived from the essential amino acids was supplied by the addition of glycine and urea. The total nitrogen was 10 gm. per day in the food. The smallest amount which could be fed daily to maintain a continuous positive nitrogen balance in the subject was considered to be the minimum requirement.

The results obtained are listed in Table V. Great variations were found in the minimum requirements, these being as much as 100% among different individuals to maintain nitrogen balance. In most subjects 0.15 gm. of L-tryptophan per kg. was sufficient to produce a positive balance, but one subject required 0.25 gm.

In the human subjects, the only D-amino acids utilized were D-phenylalanine and D-methionine. Part, but not all, of the L-phenylalanine could be replaced by the D-form. In the case of methionine the D-form appeared to be just as good as the L-amino acid. The D-amino acid was found to be unutilizable in the cases of lysine,

TABLE VI

THE RECOMMENDED DAILY PROTEIN ALLOWANCES FOR
CHILDREN AND ADULTS(Committee on Foods and Nutrition, National
Research Council) (274)

Protein Allowances (grams)					
Children			Adults		
Age (years)	Sex		Activity	Sex	
	Boys	Girls		Men	Women
Under 1	3-4/kg.	3-4/kg.	Sedentary	70	60
1- 3	40	40	Active	70	60
4- 6	50	50	Very Active	70	60
7- 9	60	60	Pregnancy		85
10-12	70	70	Lactation		100
13-15	85	80			
16-20	100	75			

valine, leucine and isoleucine. D-tryptophane was imperfectly or not all all utilized by the human subjects.

Obviously, protein allowances should be more generous for the conditions of growth, pregnancy and lactation (see Table VI).

II. COMPARATIVE NUTRITIONAL VALUES OF PROTEINS, PROTEIN HYDROLYSATES AND AMINO ACID MIXTURES

1. Dynamic Interrelations Between the Amino Acids and Proteins

Until recent years, the theory of protein metabolism in the animal in nitrogen equilibrium embodied the concept of a quota for repair and formation of tissue proteins (endogenous protein metabolism) and another quota for non-protein purposes (exogenous protein metabolism). This was a rational concept supported to some extent by the fact that certain products of intracellular metabolism (*i.e.*, creatinine) were excreted in relatively constant amount regardless of the amount of protein ingested. Amino acids were called "building blocks" for protein construction and for the repair of tissue proteins. The latter were regarded as static and permanent. In recent years this concept has become untenable. The breakdown of intracellular protein must be continually in progress to an extent far in excess of the "endogenous" quota (80, 78, 322). The employment of amino acids labeled with the heavy isotope of nitrogen, N^{15} , and sometimes with deuterium,

furnished direct evidence of a rapid interchange of amino nitrogen and carbon residues between ingested amino acids and those bound in the tissues. Furthermore, intact amino acids are continually entering and leaving tissue proteins. The proteins of liver, kidney and blood plasma and the various fractionated proteins of these tissues are found to be highly active in the uptake of isotopic nitrogen. The proteins of muscles and skin are slower, and erythrocytes quite slow in taking up N^{15} (323, 324) of labeled amino acids. Following the administration of methionine labeled with radioactive sulfur to animals, the isotope is found in the proteins of many organs, even throughout the starved animal (352).

Some years ago it was demonstrated that the daily quota of tryptophan given all at once was less effective than when given in several doses at intervals (59). Evidently the otherwise complete assortment of amino acids could not all be retained until the tryptophan became available for tissue protein formation, and quickly underwent other forms of metabolism. Further experiments of this nature with tryptophan, methionine and lysine have shown that delayed supplementation of diets lacking any one of these amino acids leads to very inefficient utilization of the dietary protein or protein hydrolysate (145). Similarly, feeding two different groups of the indispensable amino acids alternately results in very poor N utilization by rats, although good utilization ensues when the groups are mixed and fed (93).

There remains no doubt that the amino acid and protein metabolism in the body is a rapid and continual process. Amino acids are interchanged and interconverted; some protein molecules are torn down while others of their kind are being built, or they are converted into proteins of another type (321). The entire process takes place in one large system or "pool" within which it is not possible to segregate any particular form of metabolism, such as the supposed "endogenous" type.

Absorbed amino acids enter immediately into the metabolic pool. If not all materials are at hand to facilitate tissue protein synthesis, then the amino acids soon undergo catabolism and excretion. There is no storage analogous to that of carbohydrate and fat, and no "reserve" beyond the fact that organs and tissues in which protein metabolism is most active are also the ones to lose protein most rapidly during depletion.

2. Estimation of Nutritive Quality

The assay of the nutritive value of a protein is usually conducted by the study of comparative growth rates, gains in body weight or in body protein per unit of protein ingested, biological values, nitrogen balance, and tissue protein regeneration.

Recent reinvestigation of the problem of determining the relative nutritive values for growth have shown that dietary proteins may be classified as accurately by animal weight gains as by protein efficiency (49, 177), or carcass analysis or nitrogen retention (152).

The "biological value" is defined as the fraction of the absorbed food nitrogen retained in the body (264). The conduct of a biological value measurement is not so simple as this definition would indicate. A prescribed procedure must be followed and certain constants and corrections decided upon. The method depends upon the assumption, now questionable of a differential "endogenous" and "exogenous" nitrogen metabolism. It has been pointed out, however, that the concept of biological value is primarily an expression of the relationship between nitrogen balance and nitrogen intake, which, without any assumptions, can be used to evaluate proteins (260, 23). In other words, the nutritive value of a protein can be expressed as the quantity per unit of body weight, or surface area, which will suffice to maintain nitrogen equilibrium.

The measured nutritive value of a given protein varies with the level of total protein fed (49), the presence of other kinds of protein in the diet or of substances which interfere with digestion, as well as the adequacy of all other growth or health factors. It also varies with the caloric intake (81, 86, 201, 304a), since non-protein sources of energy can spare protein for energy purposes (235).

When mixed, proteins of low nutritive value, through mutual supplementation, may surpass a protein of good value. If it is true that fewer amino acids are indispensable for maintenance, as compared to growth, a protein could conceivably have a good nutritive value for maintenance and only a poor value for growth. For growing animals, the nutritive value of such a protein will vary not only with the level fed, but also with the proportion used for maintenance (48). In fact, one may never be sure that the nutritive values, however determined, hold for any but the conditions of determination. An attempt to assay a food for all the vitamins by one growth experiment would be a close analogy to the biological value determination on proteins.

On the plausible viewpoint that the quantity and quality of pro-

tein for nitrogen equilibrium is an inadequate measure of the needs of a critically depleted animal, a "rat-repletion" method has been proposed for the assay of proteins and protein hydrolysates (376). After a month or more on a low-protein basal diet, adult rats were fed an additional 9% of protein or hydrolysate in the diet and the body weight recovery noted. While a general interpretation of the data is not possible, since the preparations were not sufficiently characterized, it is concluded that only proteins or protein hydrolysates capable of an excellent rating in animal tests should be used in the treatment of sick patients (376).

Another suggested rat method depends upon the regeneration of liver protein of the fasted rat. Liver protein of the rat rapidly disappears during short periods of fasting or feeding upon diets low in protein (240). In fact, this may take place even when the animal is still growing. A very generous intake of protein is needed to promote restoration of the liver protein lost. Dietary proteins listed in decreasing order for ability to promote liver protein regeneration are: casein, lactalbumin or whole egg protein (all of equal value); liver protein, gliadin, zein and gelatin. The resemblance of this order to the general order of these same proteins for growth is striking. Complete restoration of the protein of liver did not occur until the total body protein was replaced (170). Rats on a zein diet lost liver protein, even with supplements of tryptophan or lysine, but not when both were given (222). Both are almost absent from zein.

The results furnish an excellent illustration of the role of liver protein as a *dynamic* reservoir between dietary and body proteins. While this method of assaying proteins is faster than the rat repletion method described above, it is not clear what the advantages may be of either method over a simple growth test with young animals.

In another technique for protein depletion, plasma proteins are removed by successive bleedings, followed by return of the red cells in saline or other suitable medium. This technique is called plasmapheresis, and has been widely used in studying the comparative values of proteins and amino acid mixtures in promoting plasma protein regeneration in experimental animals. Under these conditions, the regeneration of plasma proteins will be faster because there is somewhat less of the general depletion of all tissue proteins that follows from chronic inadequate protein intake. For the same reason, the regeneration will be somewhat less dependent upon the quality of dietary protein.

The results with plasma protein-depleted animals were at first confusing; it seemed that almost any amino acid mixture or protein administered could cause some plasma protein regeneration. No one amino acid can be regarded as unique in promoting hemoglobin regeneration. Although blood proteins, in general, are deficient in isoleucine, they are not regenerated efficiently unless the diet is adequately supplied with this amino acid (284). The proteins of higher quality, those with the more complete amino acid composition for growth, are now recognized as being most effective in promoting plasma protein regeneration (284). In accordance with the modern theory of protein metabolism, the proteins of blood are in dynamic equilibrium with each other and with tissue proteins (258, 248, 322). Labeled plasma proteins (prepared by feeding lysine synthesized with N^{15}) given intravenously to normal dogs disappeared from the blood at a rapid rate, so as to indicate an interchange with a mobile pool of tissue protein (135).

Casein and lactalbumin, in the form of their enzymatic hydrolysates, were compared in several ways, with the following results, respectively: nitrogen balance index, 0.80 and 1.0; growth efficiency, 2.2 and 2.7; "Strepogenin" units per gm., 5 and 4. The lactalbumin hydrolysate had a significantly higher content of lysine, tryptophan and cystine, but less methionine and valine. In plasma protein regeneration in dogs, the casein hydrolysate stimulated the regeneration of both plasma albumins and globulins, while the lactalbumin hydrolysate stimulated an increase of the albumin fraction, primarily (73). In a later study it was found that the same preparations were equally effective in restoring plasma albumin and γ -globulins, but the casein hydrolysate was superior in leading to the regeneration of "other globulins" (97). Growth experiments with small animals have for years consistently shown a superiority of lactalbumin over casein. Yet, these two proteins are equivalent for N retention in adult humans, and most probably equivalent for infants (269, 111).

In the modern concept the animal must be regarded primarily as a system of closely and dynamically interrelated proteins. A temporary deficit of one may be repaired by calling upon the other body proteins, as well as upon dietary proteins. The nutrition of a part of the proteins cannot be divorced from the nutrition of all of them. It is evident, therefore, that attempts to evaluate a dietary protein for the generation of any limited group of body proteins can hardly give much more useful data than those obtained by general growth or recovery.

Now that amino acid data on food proteins have become more extensive and accurate, it is possible to show a correlation between previously determined "biological values" for these food proteins and their amino acid contents. The biological value of a protein is modified by the content of any one indispensable amino acid which is least adequate for body requirements (266). Amino acid composition (70) and requirement data are being increasingly employed in the control of protein nutrition (for example see 157, 184, 325).

Repetition need scarcely be made of the well known facts that digestibility of a protein is important, in varying degrees to different species, and that amino acids in some proteins do not seem to be readily available. The "availability" to the rat of 10 indispensable amino acids in roast beef, peanut flour, wheat, and cottonseed flour was estimated as a difference between intake and fecal excretion. In the first three protein sources all 10 amino acids were well over 90% available. However, in the cottonseed flour, a commercial sample of unrecorded history, the availabilities of all but arginine were depressed below 90% and lysine was lowered in particular (231). On the other hand, the amino acids of cottonseed meal are as fully available to the chick as those of many other proteins (157, 28). Within reasonable limits, the analytical amino acid contents of feedstuff proteins, especially at suboptimal levels, are fully consistent with the known requirements and growth results observed with chicks (157, 28, 158, 325, 199). It would appear that, in the great majority of practical and good quality feedstuff proteins, amino acid unavailability is probably much less than 10%, and in most cases is less than inherent errors in the data.

The specific effect of methionine supplementation to raw soybeans might be construed as a lack of availability of methionine, which is actually present. The mechanism of this effect has been given much study. Proper heat treatment of raw soybean meal expedites the hydrolysis of the protein by enzymes (8, 133b, 261, 294). Methionine and lysine liberation appear to be particularly accelerated by heat treatment of soybean meal (261). This observation led to the proposition, "for optimal utilization of food proteins all essential amino acids must not only be available for absorption but must also be liberated during digestion *in vivo* at rates permitting mutual supplementation" (261). In other words, a selective hindrance to methionine liberation from raw soybean protein in the *early stages* of digestion, especially, might cause a deficiency in the ab-

sorbed amino acids which would be detrimental to their immediate use in tissue protein synthesis.

The occurrence of an enzyme-inhibiting and growth-retarding substance in raw soybeans has been demonstrated by its extraction, and its inhibiting effect on enzymatic digestion when added to other proteins (82, 168, 167), or to diets containing other complete proteins (168, 167, 219, 369a). The proteolytic inhibitor is destroyed by heat treatment (82, 74, 219). A highly purified trypsin inhibitor has been isolated from raw soybeans (232, 83).

Even after the most favorable heat treatment, soybean meal remains slightly deficient in methionine for the young chick (29a, 40). Such deficiency is also true of the isolated soybean protein, glycinin (155). Although properly-heated soybean meal can be somewhat further improved by added methionine, no supplementation of raw soybean meal with amino acids as yet reported seems to bring it entirely up to the nutritive value of the properly heated meal (40, 143, 173, 255, 294). This indicates some form of improvement in the plane of nutrition upon heating, which may well be a general result of inactivation of the proteolytic inhibitor. The above mentioned findings with the soybean have been almost exactly duplicated with the lima bean (218a).

Some investigators feel that an explanation based on inhibitor destruction is still not adequate (115, 294) since the germination of soybeans increases their apparent protein value for rats (134) without destruction of the proteolytic inhibitor (115). However, a germinated sample of soybeans which was improved for rats was not improved for chicks (254a). Predigestion with papain did not improve the biological value of raw soybean protein when it was fed to rats in the presence of the proteolytic inhibitor (115), but did cause a marked improvement for chicks (255a). The isolated crude inhibitor placed in the diets of mice reduced their gains and their efficiency of utilization of either intact casein, soybean meal, or partially enzyme-hydrolysed casein (369a, 369b). It is obvious that these questions need further investigation and explanation.

In the matter of methionine in raw soybeans there is no evidence of an unavailable chemical structure. The proper heating of soybean protein accelerates the liberation of *all* indispensable amino acids rather than of methionine in particular (99, 195a, 239a, 294). This is what would be expected following the inactivation of an inhibitor to an enzyme as general in its action as trypsin.

Another piece of evidence that methionine is not selectively in-

terfered with in raw soybeans is the fact that added methionine increases the biological retention of the methionine naturally present (133a). If there are any other detrimental effects of the proteolytic inhibitor, these must be relatively minor since adding methionine alone to raw soybeans will correct up to 90% of the growth retardation (40, 133a).

Nevertheless, in the case of a protein which contains a slight sub-optimal or barely optimal level of one or more essential amino acids, "the available level of the amino acids may be decreased by inhibition of proteolysis to the point where the first amino acid which fell below requirement levels would become the limiting factor upon growth" (168). This viewpoint has the advantage of avoiding the assumption of selective hindrance to enzymatic liberation of a certain indispensable amino acid. The relative reduction of growth of rats on casein diets to which tryptic inhibitor is added is largely overcome by adding methionine or cystine (218a). Since casein is not a fully adequate source of the total S-containing amino acids this result is in harmony with the above expressed theory.

Severe overheating of protein meals such as fish (98), meat (65), soy globulin (133c), and casein (132, 285), which contain little or no free carbohydrate, will cause the amino acids lysine, and possibly arginine and histidine, to become bound to other amino acids by new chemical linkages which apparently reduce the availability of these amino acids to enzymes and to the animal. The amino acids so bound can be regenerated by acid digestion of the heated proteins and, therefore, are not chemically destroyed. The possibility of such binding of arginine has been formerly pointed out (26). Excessive damage to arginine would be particularly serious for the nutritive value of proteins for fowls, but would be less notable with mammals.

The overheating of proteins in contact with much carbohydrates causes a second type of nutritional destruction of amino acids apparently through their reaction with carbohydrates that particularly attack lysine, arginine and tryptophan (99, 289a, 294, 348, 133c). The reaction appears to be a general one between almost any amino acid and any aldehyde (266a). This is a permanent chemical loss, that is, the amino acids attacked in this way cannot be fully regenerated by chemical hydrolysis (133b, 289a, 294), nor in the digestive tract (348).

Samples of skim milk powder, which had darkened in color and become less palatable were found to have lost significant amounts of arginine, histidine, lysine, methionine and tryptophan. Stored

samples which retained a fresh appearance and taste suffered no significant losses of amino acids (186). Partial disappearance of lysine, arginine, histidine and tryptophan in casein when heated in glucose solution has been shown to take place (289a). Under heating conditions which caused a 30 to 40% loss of cystine and lysine, the methionine in soybean meal was undamaged (133b). Overheated soybean meal protein shows a nutritive deficiency of lysine (99, 133b, 143, 255, 294, 348). This is also true of fishmeal protein (98). Since there is a nutritional surplus of lysine in these sources of protein, it is evident that the loss from overheating must be comparatively large before it is seriously felt by the animal.

Two distinct possible types of amino acid impairment on heating of food proteins have, therefore, been demonstrated. Both types of heat-induced reactions most probably result in what amounts to an actual loss of the amino acid for nutritional purposes.

A more general effect of overheating is to retard the rate of enzymatic digestion and the release of all the indispensable amino acids, rather than any one in particular (65, 98, 133b, 294). This retardation of enzymolysis is in addition to specific heat-caused binding or destruction of the more sensitive amino acids; it may be observed in the absence of any analytical loss of amino acids and appears to be very similar to the effect of an enzyme inhibitor.

The early *rate* of lysine liberation from heat-damaged casein by enzymes was reported to be affected even more than the ultimate *degree* of liberation. Thus, the lysine may have been "liberated at a rate too slow to allow effective combination with other amino acids absorbed earlier in the process of digestion (285)." However, the general rate of enzymatic casein digestion was also correspondingly reduced.

On the other hand, it may not be necessary to assume selective interference, as a result of overheating, on the enzymatic liberation of any one amino acid, any more than in the case of an enzyme inhibitor. Most probably there is first a definite nutritional loss by one or more of the heat-induced reactions discussed above. Second, there is a retardation of enzymatic digestion which serves to aggravate any deficiency which may have been naturally present or induced by the overheating. The explanation which best fits the case of accentuated methionine deficiency in raw soybean meal (168) may be extended to fit the case of lysine and other deficiencies in overheated meals. If this is true, the natural methionine deficiency in soybean meal should also become aggravated by the overheating form of enzymatic retardation. This is what has been

observed. Both lysine and methionine additions, jointly, to overheated soybean meal are required to permit even moderate rates of growth (99, 348). The possibility remains of other deficiencies, induced by the enzymatic resistance, that are secondary to those of lysine and methionine.

Modern and carefully controlled processes for heating, cooking and drying of protein meals do not seriously diminish relative availability of any amino acids, with the possible exception of cystine (65, 133b), which is not indispensable, but is of practical value in the sparing of methionine. It seems probable that reports of impaired cystine absorption may have been a result of heat sensitivity of this amino acid.

Several reports have appeared recently on the amino acid content of canned meats and fish. The drastic heating required to sterilize meats and fish in the can may be suspected of causing some amino acid damage. However, no loss of any indispensable amino acid was found in several comprehensive studies of many canned samples when compared to raw material (118a, 275a). These results are consistent with those of several earlier studies on overheated proteins of the same types, i.e., analysis following chemical hydrolysis of the sample shows no loss of any amino acid. A nutritive loss by heat-induced binding of some amino acids, as well as increased resistance to enzymolysis, have not been ruled out by these studies.

The keratin proteins are marked by their resistance to enzymatic digestion; this causes their nutritive value to be very low for most species. The digestibility of keratins such as hair, wool and feathers may be increased in several ways. Finely powdered wool and feathers fed as the only source of protein for rats are capable of supporting moderate growth when supplemented with lysine, histidine, methionine and tryptophan (314). With the same supplements, powdered hog hoofs support good growth of rats and chicks, while poorer growth occurs with powdered hog hair and chicken feathers. The more finely ground products support better growth, in general (278). It seems that enzymatic attack on keratins is expedited by increasing the surface exposed (283). The exposure to heat and oxidation during the attrition in a ball mill causes a loss of amino acids, notably tryptophan (101) and cystine (131). If properly supplemented by other proteins in practical diets, powdered hoof and horn meal is able to meet part of the protein requirements of growing chicks (338, 362).

3. Proteins and Their Hydrolysates

Frequently, during the past 10 years or more it has been reported that digests of proteins will not promote as good growth as the original proteins. However, one exception to this general finding has been reported for enzyme-digested casein (269). In an attempt to study this question explicitly, casein carefully digested by enzymes has been fed in otherwise complete diets to chicks. With casein hydrolyzed from 48 to 79%, the predigested casein is invariably inferior to the intact casein, regardless of the level of protein fed or the simultaneous presence of intact casein in the diet. This inferiority is more marked with the greater degree of digestion. The result can not be explained on the basis of destruction or deficiency of an amino acid, or of any other growth factor in the casein (349). It is noteworthy that acid-hydrolyzed casein, even when most favorably supplemented, is incapable of supporting as good growth as the intact casein with either the chick (220) or the rat (330). At the same caloric intake both enzyme- and acid-hydrolysed casein plus tryptophan were inferior to whole casein for maintaining N balance in adult humans (304a).

It has been postulated that a growth factor for a microorganism, *Lactobacillus casei*, exists in certain whole proteins and is destroyed by complete acid hydrolysis, but may be liberated from the proteins by partial enzymatic or acid hydrolysis (342, 341, 390). Preliminary evidence has been offered that this bacterial factor, called "strepogenin," is also a growth factor for mice (386). Other investigators (382) seem to agree that the diminution of the growth value of a protein upon hydrolysis must be due to the destruction of a peptide-like "growth factor." Such evidence, however, does not serve to show unequivocally that the destruction of the bacterial growth factor and the decrease of growth effects for animals are identical, and they may well be entirely separate, although roughly parallel, processes. The bacterial growth effects attributed to strepogenin are exerted to some degree by glutamine, glutathione and serylglycylglutamic acid. The latter may be a fragment or close relative of strepogenin (389).

The nature of the unique growth-promoting effects associated with crude sources of protein of animal origin (animal protein factor) has been clarified greatly by the isolation of a new member of the vitamin B complex vitamin, B₁₂. The reader is referred to a recent review for further details (349a).

The hydrolysis of protein prior to ingestion has the effect of

eliminating one slow step in the natural assimilation of proteins, *i.e.*, enzymatic digestion. When the protein is predigested all the effects of the nature of the protein and the mode of action of the enzymes are eliminated, to the extent of the completeness of the predigestion. Hydrolysis of protein before oral administration allows a more rapid absorption of the constituent amino acids, and a higher blood amino nitrogen content (223). The rate of absorption of individual amino acids from the gut of the chick is determined primarily by molecular size (223). Similar work with other species is reviewed in this paper (223).

The plasma levels of arginine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine rise when the corresponding amino acid is given by stomach tube (180). Free amino acids in the plasma are very rapidly removed by tissues, liver and kidney, especially (141). Amino acid mixtures rapidly infused into normal animals are lost in the urine to an extent of 9 to 23%, and after slow infusion from 4 to 11% (335). Practically all of the recognized amino acids have been found in urine, partly in peptide combination (318, 119, 326). The loss of amino acids in urine samples is maximal in humans about three to four hours after a meal (139). Internally, this loss is probably much sooner than indicated. Also, it must be related to the levels of the amino acids in the blood and to the length of time they are left circulating in the blood. Another observation consistent with this general concept is the fact that rats lose more amino acids in the urine when fed proteins deficient in one or more indispensable amino acids (326, 319). On purely physiological grounds, therefore, there is good reason to expect that predigestion of protein may cause some reduction in its nutritive value, such as increased loss by excretion and catabolism, quite apart from the destruction of any known or hypothetical growth factor.

4. Amino Acid Mixtures

The question of the suitability of a mixture of amino acids as a substitute for dietary protein is of experimental, practical and clinical importance. There now remains no doubt that the ten amino acids which are "indispensable" in the diet of the rat are all that are needed to permit young animals to grow, or adults to remain in positive nitrogen balance (306, 174, 302, 216). Rats can be maintained in nitrogen and weight balance when fed this group of amino acids less arginine (378). While there have been some reports of failures (18), it is most probably that in such cases the amino acid mixtures are not so complete as supposed or that there are toxic

impurities present in some of the dietary components. Also, the explanation has been advanced that bacterial synthesis of unknown amino acids, or other growth factors, in the intestine proceeds more readily when intact protein is fed as compared to the amino acid mixtures, hence the usually observed better growth (254).

Considerable quantities of racemic amino acids may be fed without seriously affecting growth of the rat (216), but the data offered do not bear sufficiently on this point to show whether or not there may have been some detrimental influence of the unnatural isomers.

Comparisons in human subjects of the proteins of several foods with equivalent mixtures of the indispensable amino acids, showed a lower "biological value" by from 10 to 40% for the amino acid mixtures (273). The authors came to the conclusion that the D components of the racemic amino acids used in these studies should be regarded as of little or no value.

When fed amino acids or hydrolyzed proteins in place of intact proteins, the animal usually decreases its voluntary feed intake. It is possible that diets containing the rapidly absorbed uncombined amino acids may cause a physiological reaction leading to a diminished appetite that is not the case with intact proteins in the diet (28). It is noteworthy that the blood amino N rises more rapidly in the former case. There is ample evidence in the literature that the too rapid forced ingestion of amino acids will make animals feel ill, and even cause vomiting. The effects of an indispensable amino acid deficiency in the diet are somewhat similar (306, 307).

The marked reduction of appetite noted with all animals during such a deficiency is probably an extreme stage of the same effect noted with complete mixtures of amino acids in the diet (28). In this case the metabolic paths for the disposal of amino acids, which cannot be removed via protein synthesis, are flooded, and the animal responds with a drastic curtailment of food consumption. When the lacking amino acid is restored to the diet, even though such addition could hardly affect the sensory acceptability of the diet, the increase in the appetite of the animal is quickly evident. The changes in weight and appetite of the chick upon removal or restoration of an indispensable amino acid to the diet may be clearly indicated within 24 hours (36, 31). More recently the same speed of response has been reported for the rat (140).

The relative efficiencies of proteins and comparable preparations of uncombined amino acids will also vary according to the level of intake. At lower levels, approximately half of optimal intakes for growth or at nitrogen balance levels (212), the efficiency of utiliza-

tion of an equivalent assortment of amino acids from any source becomes very much the same. The conflicting statements in the literature on this point are, in most part, due to the different levels of protein employed in the different studies.

There is an additional effect of uncombined amino acids which may operate to the disadvantage of an amino acid mixture in the diet as compared to a whole protein of corresponding amino acid

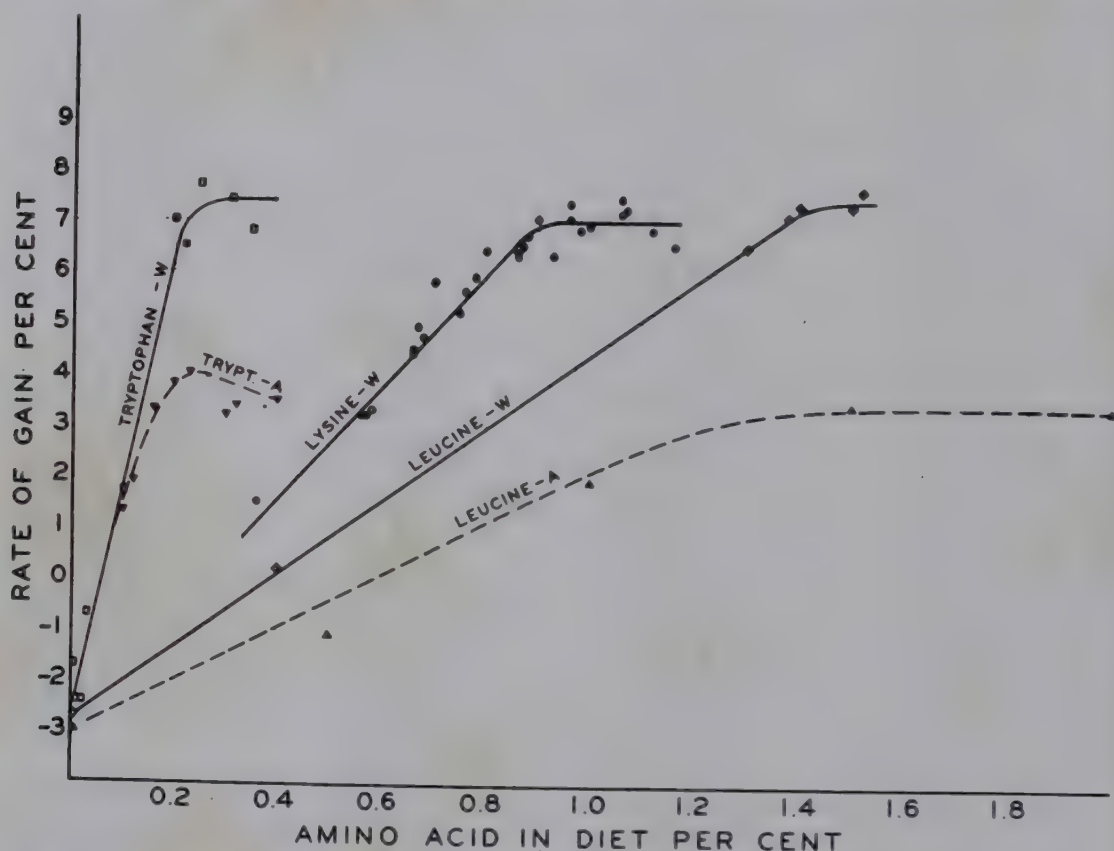


FIG. 1. The relation of the daily rate of gain of chicks to the percentage of certain amino acids in the diet. W-curves refer to whole proteins in diets, A-curves refer to amino acid mixtures or hydrolyzed proteins diets.

content. Many free amino acids have been found to increase the niacin (or tryptophan) requirement. Furthermore, excessive niacin is detoxified by combination with ornithine (113). Combination of niacin with other amino acids remains a possibility. It is not impossible that some similar, although milder, effects are felt when an amino acid mixture aimed at meeting only the normal needs is ingested.

A critical study (28) of a large body of data on chick amino acids requirements, leads to the following conclusions:

1. The total absence of any completely indispensable amino acid, or of protein, from the diet of the chick causes a loss of weight which approaches a common limiting value. This common value rep-

resents a state in which protein synthesis has completely stopped while tissue protein degradation continues. It is the true zero point of the growth curve.

2. Any rate of gain significantly more positive than the limiting value, is evidence that synthesis of protein is taking place. If this rate is still negative it is then an expression of the inability of protein synthesis to compensate for protein degradation. Growth is

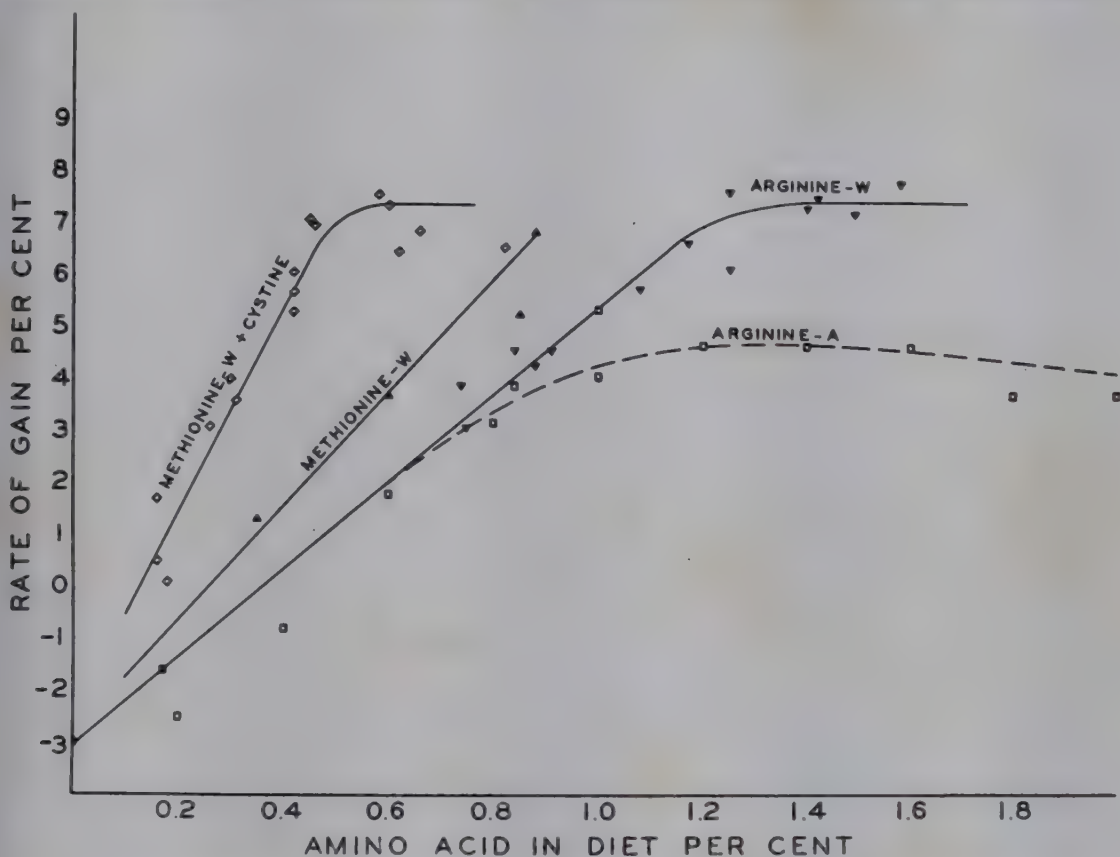


FIG. 2. The relation of the daily rate of gain of chicks to the percentage of certain amino acids in the diet. W-curves refer to whole proteins in diets, A-curves refer to amino acid mixtures or hydrolyzed proteins diets.

the result of the balance between a variable protein synthesis (dependent on diet) and a relatively constant protein degradation (independent of diet), both of which are proceeding rapidly.

3. The relation of the daily rate of growth to the level of an indispensable amino acid in the diet (at an optimal level of total protein) is substantially linear up to the optimal rate (see Figs. 1 and 2).

4. Amino acid mixtures (and hydrolyzed proteins) are less efficient than comparable whole proteins for growth, however, the optimal amino acid requirements determined with either source of amino acids agree closely.

5. For the most efficient synthesis of protein at any rate of growth

the proportions of the indispensable amino acids to the others in the diet has a relatively fixed value. This is compatible with the concept that protein synthesized by a given animal is always of the same characteristic composition, and hence requires the same proportions of constituents.

On the basis of this work an indispensable amino acid was defined as "one which must normally be obtained from the gastrointestinal tract in order that synthesis of body proteins may take place" (29).

Confirmation of the concept that growth varies with the rate of synthetic reactions as opposed to degradative reactions has been stated recently, on the basis of studies of protein formation in liver tissues of rats at various ages (142).

A study of lysine requirement of the chick at four dietary levels of protein furnished as sesame meal has brought out very clearly the fact that the most efficient utilization of the protein at any level could be attained only after the addition of lysine. Similarly, the requirements for methionine (29a) and for arginine (40a) have been found to be proportional to dietary protein levels, even when these levels greatly exceed normal levels.

The correct proportions of amino acids to each other in the diet are far more important than gross protein intake. Evidently, the body cells have little ability to select amino acids differentially from the supply offered in the blood stream. For maximum efficiency of utilization this supply of amino acids must contain certain proportions of amino acids to each other, at least of the indispensable amino acids. The proportions in the blood stream are in turn dependent upon the proportions in the diet.

III. PROTEINS, PROTEIN HYDROLYSATES AND AMINO ACID MIXTURES IN PARENTERAL NUTRITION

1. Proteins

In cases of shock, acute gastrointestinal disease, advanced starvation and severe protein allergies, it may be necessary to resort to protein nutrition by routes not employing the alimentary tract.

The only proteins, of course, which can be safely administered by such means are those which are native to the species, *i.e.*, plasma proteins from the same species and compatible blood type, or modified soluble proteins, such as gelatin, which have no allergenic reactions. Human plasma and albumin solutions are very costly and their use is practically limited to emergencies or treatment of

shock. Gelatin is such an incomplete protein that it has little value nutritionally.

2. Mixtures of Isolated or Synthetic Amino Acids

Certain mixtures of amino acids seem to be well tolerated during intravenous administration. Lack of tolerance has been found caused by too much glutamic acid (251, 361) or aspartic acid (361, 245). It also seems that an overabundance of the D-isomers (as in the racemic forms) decreases the tolerance to the amino acid mixtures (194, 109).

A mixture of ten amino acids: threonine, valine, leucine, isoleucine, lysine, tryptophan, phenylalanine, methionine, histidine and arginine, maintains nitrogen equilibrium and promotes plasma protein production in plasmapheresized dogs (247) and in dietarily protein-depleted dogs (110). Effective regeneration of plasma protein is observed in several methods of administration: oral, intravenous, subcutaneous and intraperitoneal; but the oral route appears to lead to somewhat more efficient utilization of the amino acids. There is little or no indication that the D-isomers are toxic in the amounts used. Similar results have been obtained with both dogs and humans (250).

A mixture of the ten indispensable amino acids plus glycine has been given further study with humans (366). The mixture is a bitter tasting solution of pH 5.5. This taste is due primarily to the basic amino acids and is also characteristic of hydrolysates of proteins which are rich sources of arginine and lysine. The solution was given mainly by intravenous infusion, in amounts up to 3 liters per day, containing N equivalent to 95 gm of protein. This is three times the balance requirement for a normal healthy human adult. Nausea and vomiting were the only frequently noted ill effects, and these were less troublesome at slower rates of infusion. A blood amino N of over 20 mg % consistently accompanied the ill effects. A dose of 400 ml per hour of 8% solution was usually tolerated. The great bulk of the amino acids was destroyed, as indicated by a marked rise in blood urea and in N excretion. This was so rapid as to contraindicate the use of the amino acids in cases of renal damage. Patients who tolerated the mixture were able to replenish body protein, to maintain a more favorable N balance and to recover somewhat sooner than control patients (366).

The large waste of N is explained as being due to both a heightened catabolism and anabolism. On the other hand, it should be

recalled that the mixture of amino acids contained only about half those needed for tissue building; the patient was forced to synthesize the amino acids not provided. This inevitably would result in wastage of the amino acids infused; moreover the synthesis requires time, during which the infused amino acids have no harbor and are completely exposed to channels of loss and destruction. The synthesis of tissue protein from such an ingested incomplete mixture of amino acids will obviously be limited by the rate of formation of the most slowly synthesized amino acid needed to form tissue.

3. Protein Hydrolysates

Hydrolysates of proteins are being more commonly used when the protein nutrition is the prime consideration. Amino acids are still very expensive, hence the interest in the cheaper protein hydrolysates. Furthermore, a hydrolysate made from a good quality protein is automatically a well-balanced source of amino acids. The hydrolysis of a protein need not be carried all the way to completion in order to destroy allergenic properties or the danger of causing anaphylactic shock (270).

Not many years ago, it was possible to give only glucose, salts and perhaps vitamins by parenteral means. Now it is feasible to administer a more nearly complete parenteral diet for some time, to the great advantage of the patient. Due to the fact that all nutritional factors are not yet known or well understood, it is advisable to revert to an oral diet of natural foods as soon as the condition of the patient permits.

Protein hydrolysates are well utilized by parenteral routes. The nausea and vomiting reactions caused by glutamic and aspartic acids may be experienced if these amino acids are present in relatively high levels, as in casein hydrolysates. Even if there is some lack of absolute nutritional value due to moderate imbalance or deficiency, or physiological causes, the speed and certainty with which such suitably administered preparations become effective are their greatest advantages.

Enzymic partial hydrolysates of protein have compared favorably with amino acid mixtures on the basis of nitrogen retention in protein depleted dogs fed intravenously (110). Rats will grow when obtaining all amino acids from an enzymatic protein digest given subcutaneously (193). A similar preparation given intravenously to dogs effected satisfactory plasma protein regeneration (100). An enzymic digest of casein given parenterally to young men was

efficiently utilized with respect to the free amino acids contained therein, but the peptides in the preparation were only poorly retained by the kidneys (96). Some difficulties have been encountered due to the presence of undesirable substances in the crude sources of enzymes employed to make these digests and of allergenic residues remaining from insufficiently digested protein.

Acid hydrolysates of proteins have been extensively tested. These hydrolysates are usually fortified with added DL-tryptophan, to replace the natural tryptophan destroyed. Dogs have been maintained in positive nitrogen balance on purified diets, wherein the sole amino acid source was completely-acid-hydrolyzed protein (plus added tryptophan) given either orally or intravenously (211, 133, 295).

Complete acid hydrolysis of the protein is not necessary. Partial acid hydrolysis may lead to less extensive loss of tryptophan (370, 144). These partial acid hydrolysates are well tolerated by vein and efficiently utilized for maintaining nitrogen balance, and for plasma protein regeneration (295, 144). The quantity of tryptophan remaining in these acid hydrolysates will be a primary factor controlling their nutritive value. The requirements of animals, so far as known, would indicate that at least 1% of the protein in the diet should be tryptophan while for maintenance perhaps 0.5% will suffice.

IV. PROTEIN NUTRITION IN VARIOUS NON-PATHOLOGIC AND PATHOLOGIC CONDITIONS

The proteins of blood are usually considered in two fractions, *i.e.*, hemoglobin and plasma. In protein starvation the plasma proteins are among the first to be diminished.

Low plasma protein is called "hypoproteinemia," but this term is quite commonly used to indicate general depletion of body proteins. Hypoproteinemia accompanies a wide assortment of non-pathological, clinical and pathological conditions. Some of these conditions will be discussed briefly.

1. Non-Pathologic Hypoproteinemia

a. Protein starvation

Insufficient intake of food proteins of good nutritive value will lead, ultimately, to hypoproteinemia (364). This has been particularly exemplified among the malnourished prisoners and the starved populations of World War II (313). The prevalent deficiency in

caloric intake was probably a contributing cause to the protein deficiency since protein may be diverted to energy production when necessity dictates. Carbohydrates have a "sparing action" on protein at least to the extent of minimizing protein destruction for energy purposes.

Frequently associated with the hypoproteinemia is a nutritional edema which may reach a marked stage when the plasma proteins are yet only slightly lowered (214). This is a swelling or accumulation of fluids in interstitial spaces, which results from an alteration of the osmotic balance of blood and tissue. However, it is known that many other factors, such as salt intake, water intake, posture, exercise and environment, will affect the incidence of edema. Persistent plasmapheresis of the dog causes an edema, as might be expected.

A concomitant condition of anemia following protein depletion is due to a reduced synthesis of the protein moiety of hemoglobin (165, 365, 114). Clinically, this form of nutritional anemia has been found very often associated with edema and hypoproteinemia. Experimental negative nitrogen balance in humans was found to induce a lowering of total blood protein, plasma albumin and plasma globulin at an early stage (176). All these conditions are alleviated when sufficient quantities of good quality proteins are administered thus fostering the regeneration of blood proteins and the normal osmotic balance.

A severe deficiency of an indispensable amino acid produces the same condition as general protein starvation. A deficiency of cystine, for example, has no effect on blood elements, while a deficiency of methionine or tryptophan causes a lowering of blood proteins and a hypochromic anemia (13). Corneal vascularization in the rat may be caused by a gross protein deficiency or merely by deficiencies of certain amino acids (61, 166, 350).

Almost as certainly, although usually more slowly, a chronic deficiency of a vitamin which is concerned with amino acid metabolism, *i.e.*, ascorbic acid, pyridoxine, niacin, results in such symptoms of hypoproteinemia as anemia, edema, lowered antibody production and reduced enzyme secretion (116).

For some years there have existed indications that various B-complex vitamins, *i.e.*, thiamine and riboflavin, were involved in protein metabolism. Where such observations were based upon body weight, N retention, fat deposition and similar non-specific measurements, the evidence cannot be construed as anything more than an interrelation of the vitamin to protein metabolism via al-

tered growth rate or well-being. Where, however, the effect of the vitamin has been shown on some distinct phase of intermediary metabolism of one or more amino acids it is safe to conclude that the vitamin is important to general protein metabolism or to a particular phase of amino acid metabolism.

In the extreme stage of starvation, people often lose the ability to retain food because of vomiting or diarrhea, or become unable to swallow food. Even without these complications, complete failure of the gastrointestinal tract may ensue. However, these supposedly irreversible states of inanition have been treated successfully by intravenous nutrition employing amino acids or protein hydrolysates, especially when accompanied by glucose and other nutrients to simulate more closely a complete diet. By such means, persons near death from starvation have been revived to the point where oral foods could be utilized (230). It may be pertinent to point out that one effect of the injected amino acids could conceivably be a stimulation of enzyme synthesis, since enzymes are also proteins and, therefore, cannot be formed until amino acids are available, nor can dietary proteins be utilized unless enzymes are available to act upon the proteins.

b. Obstetrics and pediatrics

Hypoproteinemia is commonly found during pregnancy, due to the drain on plasma and tissue proteins to meet the needs of the fetus (259). Alterations in blood volume, impaired digestion, and vomiting are additional causes of hypoproteinemia in this condition. The general accompaniments are also common, *i.e.*, edema (45) and anemia (63). Complications such as the toxemias and eclampsia (64) are more prevalent in association with hypoproteinemia. Elevated protein intake together with a provision of an otherwise excellent diet are unquestionably proven to be desirable during pregnancy, not only from the standpoint of the welfare of the mother, but also for the prenatal influence on the welfare of the young following birth (356, 373). The increased protein requirements of these periods have caused the Food and Nutrition Board of the National Research Council to recommend increased allowances of protein (275) (see Table VI).

In the first two years of life, blood protein levels are usually lower than in later years, and are still lower in premature infants. The incidence of nutritional edema in children is higher in the early years of life. Inadequate intake of protein is particularly serious to the young since the needs for growth are high. Poor dietary habits,

diarrhea, vomiting and protein allergies often seriously reduce the intake and assimilation of protein.

Protein hydrolysates given orally are useful in maintaining protein nutrition of infants in the face of serious allergies to the common infant food proteins, *i.e.*, milk and eggs (334, 183). The subject of protein in pediatrics and pregnancy has recently been reviewed (239).

c. Geriatrics

Protein deficiency in aged individuals may be manifested by mild anemia and lack of vigor. Poor appetite and digestion, as in achlorhydria, constipation, and putrefaction may impair the utilization of protein. Reduced caloric needs automatically tend to diminish protein intake. The provision of ample protein during this period of life enhances the general welfare. Protein concentrates and hydrolysates are particularly useful in maintaining optimal protein intake at minimum bulk of diet for older people.

2. Pathological Hypoproteinemia

a. Trauma

The fact that in injuries, surgery, and certain disease states there is a surprisingly large loss of nitrogen has been given increasing attention. Losses of weight of patients after operations, trauma, fractures, infection and burns may amount to one or more pounds per day (242), including a heavy nitrogen loss (105). A large part of this loss is by urinary excretion or by loss of blood, but significant losses may also be represented in the exudates from burns or other damaged tissue, and pus formation. Even minor operations and injuries are capable of causing a high rate of nitrogen loss (133).

At the height of protein catabolism ingested proteins and amino acids may appear to have very little effect on reversing the negative balance. After tissue injury from trauma or surgery there is a decrease in the blood amino N which is roughly proportional to the severity of the damage, and which rises again during recovery (253).

These losses of protein are detrimental to the recovery of the patient when severe enough to cause hypoproteinemia. It is rather hopeless to attempt to reverse the negative nitrogen balance of such subjects by giving only one indispensable amino acid, yet this attempt has repeatedly been made. In any event, the increase of the intake of nutritionally complete proteins and amino acids, when

the condition of the patient will permit, is generally recognized as desirable in promoting resistance, repair and recovery. Recent reviews will provide further detailed discussions of protein nutrition of surgical and burned patients (234, 243).

The effect of previous diet on the resistance of rats to a standardized trauma induced by a drum method was carefully studied. Complete absence of protein from the diet greatly increased the mortality rate of rats subjected to trauma, especially of young rats. Young rats more rapidly regained a resistance to trauma when the protein intake was restored. Levels of protein that did not support growth nevertheless imparted considerable protection. Certain incomplete proteins such as zein and gelatin were approximately as effective as casein or wheat germ in protection against the shock of drum trauma. Approximately half as many days of normal protein feeding was required to correct the effects of a period of depletion of protein. No individual amino acid imparted a definite protection, when added to a protein-free diet. A mixture of all the indispensable amino acids was more effective under similar conditions. In general, the results suggested that the protective action was related to the protein nitrogen intake rather than to any amino acid pattern. The fact that on a nonprotective diet rats could develop a resistance to periodically repeated trauma indicated that body proteins could be employed for the generation of protective agents (357).

The physician and the surgeon are now becoming increasingly concerned with the plane of protein nutrition in the preoperative, as well as postoperative state, since it has been made evident that the hypoproteinemic patient is a comparatively poorer surgical risk. Herein lies one of the most important effects of the intravenous administration of blood plasma, aside from the treatment of shock, since a heightened blood plasma level will augment the supply of readily available protein for repair processes. However, plasma is not essential for this particular duty and its place may be taken by any similar non-toxic, non-allergenic and non-pyrogenic preparation rich in the indispensable amino acids.

An estimation of hemoglobin or plasma or serum protein levels is not, by itself, a sufficiently reliable diagnostic criterion. In a rather large variety of conditions such as trauma, chronic infections, tumors, ulcers, hepatic disease and malnutrition, a deficiency in total circulating hemoglobin may be present, although disguised for some time by a contraction in blood volume. A critical evaluation of the status of a surgical patient with regard to protein must include optimum as compared to actual body weight and direction

of change in body weight, plasma total protein and albumin level, plasma volume, and nitrogen balance (242).

b. Ulcers

Peptic and duodenal ulcers, enteritis and ulcerative colitis are frequently associated with low protein diets and hypoproteinemia. Bleeding from such lesions may lead to further protein depletion. A most effective treatment of such diseases has included a high intake of hydrolyzed protein. Relief from distress, gain in weight and strength, and healing were prompt results from the oral administration to gastric ulcer patients of protein hydrolysates (42, 272, 106). Surface ulcers are also highly correlated with hypoproteinemia. Healing is often initiated and accelerated when the protein intake is amplified through the addition of hydrolysates or amino acid mixtures (42, 272).

c. Liver disease

During protein depletion, the liver loses protein and weight, but becomes richer in fat. This may ultimately result in cirrhosis and necrosis (280, 148). The injured liver cannot then so well carry out its function of manufacturing blood proteins, and a vicious cycle commences. Other hepatic diseases such as hepatitis usually interfere with protein metabolism in the liver, causing an accelerated loss of nitrogen. Treatment of these conditions includes a high plane of protein nutrition (343). (See pages 600, 616.)

d. Kidney disease

Certain forms of kidney diseases are often accompanied by poor appetite, proteinuria, edema and other signs of protein deficiency. These patients need an elevated intake of protein or protein hydrolysate to compensate for long-continuing losses or to expedite any clinical improvement (343).

e. Diabetes

In uncontrolled diabetes, large quantities of nitrogen are lost through the attempt of the organism to convert amino acids into sugar. There may be a protein deficit even with a good intake of protein. Ulcers, wounds and other lesions may prove difficult to heal because of the existing hypoproteinemia. Ample protein in the diet, even after proper control of the disease has been established with insulin, is indicated to favor recovery from the protein deficit resulting from the large metabolic losses.

f. Fever

Rickettsial spotted fever disease causes marked disturbances in mineral and protein metabolism, associated with damage to blood vessels, edema of lungs, brain and skin, a fall in blood proteins which may lead to a circulatory collapse, and a high excretion of N. Unless circulatory collapse is present, a high-protein, high-carbohydrate, high-vitamin diet is the most important supportive therapy. If necessary, the dietary protein is reinforced by liquid protein concentrates taken orally (118).

g. Hyperthyroidism

The heightened metabolism of hyperthyroidism increases the requirement for protein by virtue of accelerated losses, and may lead to hypoproteinemia if not corrected or compensated (343).

h. Cancer

Cancer of the gastrointestinal tract or the liver is commonly accompanied by hypoproteinemia; hence the welfare of patients with these conditions requires particular attention to the protein intake (242).

i. Immunity

A further general consequence of hypoproteinemia is the more recently appreciated effect upon the blood globulin fraction. It is suggested that one of the γ -globulins is the precursor for the antibodies (94). The hypoproteinemic individual has definitely less resistance to infections. In hypoproteinemic animals the formation of antibodies is much reduced (94, 92). It has been further shown that hypoproteinemic rats have a significantly lowered ability to elaborate hemolysin after inoculation with foreign erythrocytes (375, 377). Mice seem more susceptible to infections when the quality or quantity of protein is diminished in their diets (363, 316). A high-protein diet is particularly valuable for the production of hyperimmune sera in rabbits (145a).

The blood cells which are primarily responsible for the destruction of invading organisms have been shown to vary in activity and in number with the protein intake. The phagocytic index of mice increased over the range of 6 to 36% of casein in the diet (104). The leucocyte counts, leucocyte activity and agglutination titer of rats on a low-protein diet were reduced to a third of those seen in rats on a well supplemented diet (62).

The hypoproteinemic chick shows a marked decrease of serum

protein on diets below 7% protein. The resistance of such chicks to experimental infections with avian malaria is strikingly different, depending upon the protein level in the diet. A protein deficiency which is not severe enough to cause a distinct lowering of total serum protein, still has a marked effect on the course of the disease. On a 32% protein diet, chicks do not die from the disease and show the greatest ability to clear the parasites from the blood stream (331). If such effects of protein intake upon resistance and immunity prove to be general, the consequences for medicine and human health are tremendous.

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Chapter XI

THE CHEMISTRY OF ANTIBODIES

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I. INTRODUCTION

THE PRIMARY experience of the immunologist is the observation that when an animal, such as a rabbit, is injected parenterally with any of a variety of materials, termed *antigens*, there appear in time in the circulation of the animal new protein molecules, called *antibodies*, which have the remarkable capacity to react with a high degree of specificity with the material used in the injection, or with related materials.

Within a decade after von Behring and collaborators showed conclusively that antitoxic immunity was founded on a principle transferable with immune serum, it became known that non-in-

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fectious and non-poisonous materials such as erythrocytes and egg albumin likewise were capable of engendering specific antibodies in animals. In this way, the study of immunity reactions lost its original close connection with disease and immunochemistry was born as a science in its own right.

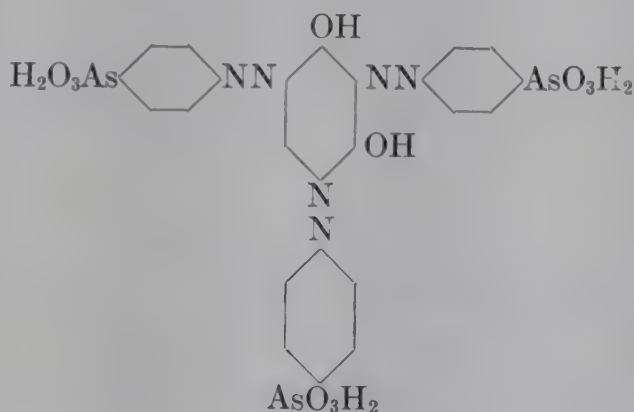
Immunochemistry is chiefly concerned with the basis of antigenicity, with the physico-chemical properties of antibodies and the structure of the antibody combining site, with the mechanism of reaction of antigen and antibody, and the role of certain non-specific materials in the reaction, and with the detailed process of formation of antibody.

The application of the principles of chemistry to immunological problems (as well as the application of immunological principles to chemical problems) has become so extensive in the past decade that it now becomes impossible to review critically the entire field of immunochemistry within a single chapter. Since the present volume is concerned chiefly with protein chemistry we shall limit the discussion to the chemical nature of induced antibodies (as opposed to the so-called natural antibodies) and we shall disregard the other half of immunochemistry—the chemistry of antigens—except in the following brief discussion, which is necessary for a clear understanding of antibody formation and antibody structure.

It has been definitely established, chiefly through the extensive work of Landsteiner and collaborators, that the ultimate basis of the specificity of antigens is to be found in their stereochemical structure. An antiserum characteristically reacts most strongly with the homologous antigen, that is, the antigen used in the production of the antiserum. This reaction is not, however, exclusive, and materials which have a structural relation to the antigen also react with the antiserum, the extent of *cross-reaction*, as it is called, depending on the degree of their structural resemblance to the antigen, or, more precisely, to the portion of the antigen molecule which is involved in the combination with antibody.

The establishment of the chemical basis of the specificity of antigens followed the discovery that it is possible to confer a new specificity on a protein antigen by treating it chemically with a variety of materials, the most common practice being to couple the protein with a chemically defined organic group such as arsanilic acid by means of an azo linkage to the tyrosine and histidine residues, and possible other residues, of the protein molecule. The antiserum to such a conjugated protein is found to contain separate antibodies to the unaltered carrier protein, to the conjugated group, and to the

structural complex involving the conjugated group and the adjacent portions of the carrier simultaneously (80). It is customary to examine such a complex antiserum for antibodies specifically directed toward the conjugated group by means of a test antigen in which the synthetic organic group is coupled with a heterologous protein carrier. In this way the reaction of antibodies against the original protein carrier is avoided. In suitable cases the reaction between the antiserum and the conjugated group (prosthetic or haptenic group) may be detected by mixing the serum with a relatively simple organic material which is structurally related with the antigen. Thus, the antiserum prepared by the injection of phenylarsonic acid-azosheep serum into rabbits forms a specific precipitate when mixed either with phenylarsonic acid-azosheep serum, phenylarsonic acid-azoalbumin, or simple dye antigens containing two or more haptenic groups, *e.g.*,



The latter material is not, however, capable of engendering antibodies in rabbits.

Furthermore, if phenylarsonic acid, $\text{C}_6\text{H}_5\text{AsO}_3\text{H}_2$, is added as the sodium salt to mixtures of any of these materials and the antiserum, the reaction is inhibited. This is a true competitive inhibition, for the extent and rate of the reaction depend on the relative concentrations of the precipitating and non-precipitating antigens and the final equilibrium is not affected by the order of mixing the reagents.

We have, therefore, several categories of antigens:

1. Complete (or functional) antigens: These are materials which are able both to elicit antibody production and to combine and precipitate specifically with antibody (*e.g.*, ovalbumin).

2. Partial antigens: a. *Precipitating haptens:* These are materials which precipitate specifically with antibody but are incapable of

eliciting its production (*e.g.*, the simple multivalent dye shown above).

b. *Inhibiting haptens*: These materials combine specifically with antibody, but they do not precipitate with it; nor do they promote antibody formation (*e.g.*, phenylarsonic acid).

For more complete discussions the reader is referred to Landsteiner's excellent review (80), which covers the field of immunochemistry thoroughly with an unbiased criticism of the literature; Boyd's textbook of immunology (11), and recent reviews by Kabat (73) and Treffers (136, 137).

II. DETECTION OF ANTIBODIES

The presence of antibodies in a serum may be demonstrated by a variety of techniques, with the choice among them depending to a large extent on the nature of the antigen which is being considered. The methods of antibody detection fall naturally into two categories, namely, those involving test tube reactions, *e.g.*, precipitation, agglutination, and complement fixation, and those involving biological reactions, *e.g.*, neutralization, hypersensitivity, and immunity.

1. Serological Methods

Precipitation. The precipitation which occurs when an antiserum is mixed with the homologous, molecularly dispersed antigen (complete antigen or precipitating hapten), under suitable conditions with respect to pH, temperature, electrolyte concentration, and especially the relative concentration of antigen and antibody, is perhaps the most widely studied immunochemical reaction. The most critical variable in the determination of the extent of the reaction is the relative concentration of antigen and antibody. The antibody which takes part in this reaction is called a *precipitin*. Thus, if a varying amount of antigen is mixed with a constant amount of antibody (or antiserum), a maximum precipitate will develop in one of the mixtures and the ratio of antibody to antigen in this mixture is referred to as the *optimal ratio*. (When testing dilute or "weak" antisera one must use correspondingly dilute solutions of antigen.) When more or less than the optimal amount of antigen is added to the given amount of antiserum, the amount of precipitate is less. As the amount of antigen is increased beyond the optimal concentration, a region, the *inhibition zone*, is reached in which the amount of precipitate is reduced and finally goes to zero in extreme antigen excess. Antigen combines with antibody in this region, but

some or all of the complexes which form are soluble. It is also possible to dissolve a specific precipitate by adding to it an excess of antigen. A typical curve showing the dependence of the amount of precipitate on the amount of antigen added to a constant amount of antibody is given in Fig. 1 (Curve I).

The inhibition phenomenon is also observed in the region of antibody excess in systems involving diphtheria toxin, ovalbumin,

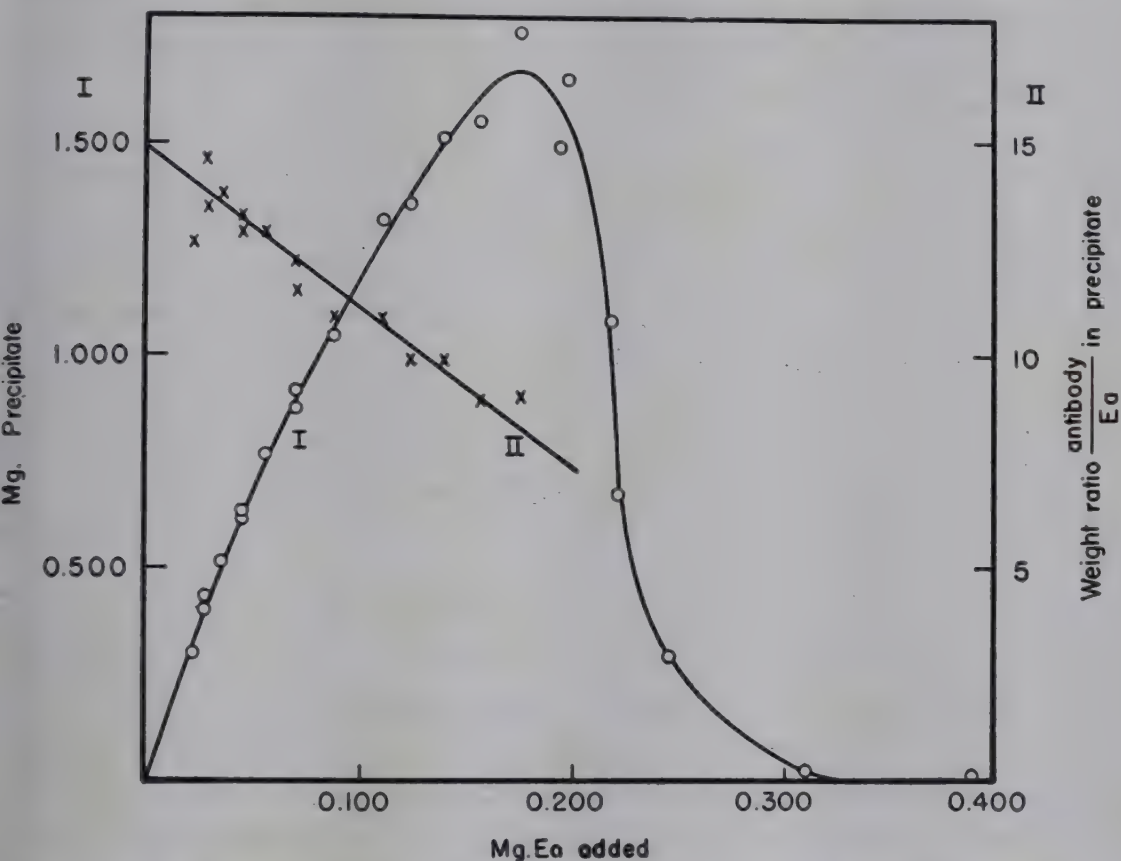


FIG. 1. The reaction between ovalbumin (Ea) and rabbit antibody. Curve I, the amount of precipitate as a function of the amount of antigen added in constant volume to a constant amount of antibody. Curve II, a plot of the weight ratio of antibody to antigen in the precipitate against the amount of antigen added. Data of R. Ballentine (7).

or hemocyanin and the corresponding horse antibody. However, in other systems involving horse antibodies against pneumococcus polysaccharides, or any of the rabbit antibodies which have been studied, there is no evidence at present of a true antibody-excess inhibition in the precipitation reaction. However, antibody-excess inhibition is often observed in the agglutination reaction.

The possibility that in a given test of an antiserum the ratio of antibody to antigen may be inappropriate for the development of precipitate may be circumvented by means of a simple test known as the *ring test*. In this test a portion of antigen solution is layered

over a portion of antiserum in a small tube and the interface is observed. Regardless of the actual concentrations of antibody and antigen in the respective layers (within rather wide limits) the counter-diffusion of reagent molecules across the interface leads at some level to conditions of concentration which are compatible with precipitation. (In carrying out a ring test it is important to use clear solutions and to set up adequate controls.)

It is appropriate at this point to consider some interesting qualitative features of serological precipitation. When a soluble precipitating antigen such as hen ovalbumin is mixed with its antibody, the reaction which occurs under suitable conditions is manifested by a succession of events which are visible to the naked eye. After a relatively short period the mixture becomes noticeably opalescent; the opalescence increases with time and presently it is possible to discern numerous minute particles of precipitate. The particles grow larger, apparently by aggregating with one another, for their number becomes noticeably reduced, and they begin to sediment to the bottom, leaving often a relatively clear supernatant fluid.

The question which we wish to consider briefly is the nature of the mechanism by which a precipitate changes with respect to quantity and form. It is generally agreed that the initial reaction between molecules of antigen and antibody is highly specific and is based on a complementary stereochemical relation between portions of the antigen and antibody molecules. The question is, does the formation of large visible aggregates of antigen and antibody molecules depend on the continued operation of a serologically specific mechanism, acting between the specifically formed primary molecular complexes, or do these complexes aggregate further by a non-specific mechanism which is not dependent on the formation of new specific links?

Long ago Bordet noted that the specific agglutination (clumping) of cholera vibrios by antiserum failed to occur in the absence of electrolyte. It could be shown that the vibrios combined with antibody under these conditions, since the supernatant fluid obtained by centrifuging off the cells was found to be depleted of antibody. Furthermore, when electrolyte was added to such a stable suspension of vibrios in antiserum, agglutination occurred promptly. Considering the analogy with the flocculation of stable colloids by electrolyte, Bordet concluded that specific agglutination occurred in two steps: a primary step in which the cells combined specifically with antibody (became sensitized) and a secondary non-specific step in which the sensitized (unstable) cells aggregated when electrolyte

was present. This two-phase theory became widely accepted and was later extended to serological precipitation (14, 38, 154), so that the latter reaction came to be described as a specific primary combination of antigen and antibody molecules, followed by a secondary non-specific aggregation of the initial molecular complexes.

In the years following 1935, it came to be recognized that an alternative formulation was possible, according to which the formation of macroscopic particles of precipitate was ascribed entirely to the continued operation of the specific mechanism by which the primary molecular complexes were formed (93, 54, 112). This alternative formulation, which has come to be known as the framework (lattice, alternation, etc.) theory of serological precipitation, was based on the likely supposition that molecules of antigen and antibody contain more than one reactive group and that, accordingly, each is able to attach itself to several molecules of the other sort. If each of the reagents has at least two combining groups, and if one of the reagents has at least three, then it should be possible to build up a framework of antigen and antibody molecules. It is generally accepted that many of the ordinary precipitating antigens have more than two combining groups, and there is evidence (see below) that some antibodies have at least two. The explanation, according to the framework theory, for the failure of the cholera vibrios to agglutinate in the absence of electrolyte is then that the similarly charged particles (sensitized bacteria) repel each other strongly enough to overcome the specific attraction, so that a given bivalent antibody molecule may not combine with two bacterial cells simultaneously and thus fails to provide the links needed for agglutination.

The appearance of the second school of thought gave rise to an active controversy which has been continued to the present day, and which, in the tradition of healthful scientific controversies, has led to the accumulation of numerous interesting serological data. In recent years the chief positive evidence for the non-specific theory has been provided by Hooker and Boyd (65). They selected two non-cross reacting precipitation systems and determined the time required for particulation, *i.e.*, for the formation of particles discernible by the naked eye, in each system. Having adjusted the two systems so as to make the separate particulation times identical, they proceeded to determine the particulation time of a mixture of the two systems prepared in such a way that each reagent was present in the same concentration in the mixed and unmixed systems. They noted that the particulation time of the mixture was con-

siderably reduced, *i.e.*, the mixed systems particulated faster than either of the unmixed systems, and from this they concluded that the two systems had interacted with each other and, accordingly, that a non-specific mechanism had a major role in the development of a serological precipitate in the secondary phase. However, their conclusion applied strictly only to the terminal period of the total process and they were not justified in extending this conclusion to the earlier period of precipitation. Moreover, their experiments were concerned chiefly with observations of the change in form of a precipitate which had already developed and had little to do with the question of the mechanism by which the precipitate developed in the pre-flocculative period.

On the other hand, support for the framework theory of serological precipitation has been provided by numerous experiments on the specific precipitability of a variety of synthetic haptens (84, 115, 66, 10). The results of these experiments, when interpreted in terms of the serological valence of the haptens used, on the whole indicate that the possibility of framework formation is a prerequisite for precipitation but is not necessarily sufficient by itself to bring precipitation to completion. From their design it is not possible for these experiments to exclude the participation of non-specific mechanisms in the development of a precipitate.

It is doubtful, therefore, if the two opposing schools were ever in real conflict, for it is apparent that each was concerned chiefly with the support of its favored theory and less with the exclusion of the opposing theory, so that the present total of evidence supports the conclusion that both specific and non-specific mechanisms participate in the visibly manifested period of precipitation. Recently, an experimental reconciliation of the opposing theories has been provided by Lanni (89) which is based on the observation that the relative participation of specific and non-specific mechanisms in the so-called second phase of precipitation depends on the time at which the process is observed as well as on the ratio in which antibody and antigen are mixed. With the use of a turbidimeter it was shown that the process of precipitation was mediated by a highly specific mechanism in the period preceding flocculation and that a non-specific mechanism was detectable experimentally only in the terminal period of this process, when macroscopic particles of precipitate were being formed from microscopic aggregates.

The terminal participation of a non-specific mechanism was especially noticeable in the region of antibody excess, and minimal in the region of antigen excess. While the actual manner of intrusion

of non-specific forces in the precipitation process was not elucidated, it was suggested that one feature of the non-specific mechanism may be the trivial entanglement of one specifically formed aggregate with another. Studies of precipitation under a darkfield microscope led to the suggestion that the total process could be effectively described in terms of two partial processes; namely, the formation of microscopic particles of specific precipitate, called "seromicros," and the aggregation of these particles. The experimental results supported the conclusion that these elementary particles are formed by a highly specific mechanism; moreover, that their primary aggregation is likewise specific, and that non-specific processes are involved significantly only in the further aggregation of the primary aggregates. Some of these conclusions are still somewhat tentative and further experiments will be required to establish them finally.

We feel that it is unfortunate that so little attention has been given in the past to the study of the precipitation process in its early period. The relative instability of the developing precipitate has so far been considered an obstacle to such a study, but it should now be regarded as a challenge, for it seems certain that, as more techniques are brought to bear, much that is of general interest will be revealed.

A discussion of some of the quantitative features of precipitation will be found under another heading.

Agglutination. When the antigen is present as a constituent of the surface of a particle such as a bacterial cell or a red blood cell, a suspension of these particles is clumped or *agglutinated* by the antiserum. The antibody which participates in this reaction is referred to as an *agglutinin*.

It is well to emphasize at this point that the terms precipitin and agglutinin are operational terms, reflecting chiefly the state of dispersion of the antigen to which the antibody is added (much less antibody being required to produce visible aggregation of large antigenic particles such as cells than precipitation of molecularly dispersed antigens). The notion that the same antibody may participate in different serological reactions is embodied in the *unitarian* hypothesis as stated by Zinsser (155). According to the unmodified hypothesis, a single pure antigen engenders only one variety of antibody and this antibody is able to precipitate the molecularly dispersed antigen, agglutinate particles (cells) containing the antigen at their surfaces, prepare (opsonize) cells for phagocytosis, protect animals against virulent homologous microorganisms, etc. In recent

years it has come to be recognized that the antibodies against a single pure antigen are not all alike (see discussion of heterogeneity below), but the notion of the diverse functional capacity of antibody is still retained.

A recent example of the intimate relationship between precipitation and agglutination was provided by Pressman, Campbell, and Pauling (122), who coupled arsanilic acid groups to rabbit erythrocytes and obtained a typical agglutination reaction between the arsanilic-azoerythrocytes and serum from rabbits immunized against arsanilic-azosheep serum. The same antiserum forms precipitates with molecularly dispersed conjugated protein antigens containing arsanilic acid groups.

Complement fixation and lysis. When sheep red blood cells are allowed to react with serum from rabbits immunized against sheep red blood cells, the cells, in addition to agglutinating, may undergo further changes so that hemoglobin is liberated. Such cellular lysis requires in addition to the antibody the cooperation of a second factor which is relatively non-specific, is not increased during immunization, and can be found in the fresh serum of a variety of animals. Since this lytic factor, which is referred to as *complement*, enters into a large number of antigen-antibody complexes, there is provided a method of detecting immune reactions, which involves the addition of a standard amount of complement-containing serum (usually fresh guinea pig serum) to the mixture of putative antigen and antibody. If an antigen-antibody reaction takes place some or all of the complement is "absorbed" by the reaction complex, and upon the subsequent addition of sheep cells which have been sensitized with antibodies against sheep cells (amboceptor) lysis may be reduced or absent.

On the other hand, if no antigen-antibody reaction takes place in the original test solutions then the complement remains free, and upon addition of sensitized cells maximal lysis occurs. This property of fresh normal serum is quickly lost at 56° C. or within several days at ordinary temperatures, although material dried from the frozen state retains its activity for some time. Four functional components (C'1, C'2, C'3, and C'4) which are associated with serum globulins, and comprise about 1.0% of the total serum protein, make up the complement activity of serum. The chemistry of these components is little known (39) and nothing is known of the actual mechanism of their involvement in antigen-antibody reactions. The specific lysis of red blood cells apparently involves only an alteration in the molecular structure of the cell membrane, with

a consequent increase of permeability with respect to hemoglobin, and not a rupture or dissolution of the membrane. On the other hand, specific lysis of some bacteria is manifested by the fragmentation of the bacterial cell. The fixation of complement by other bacteria and their antibody is not accompanied by any apparent change in the integrity of the cell membrane.

Although complement undoubtedly plays an important role in defense mechanisms and perhaps hypersensitivity, its instability, variability, and complexity detract from the usefulness of complement-fixation as a tool for the quantitative study of antigen-antibody reactions.

2. Biological Methods

Although biological methods for the detection of antibodies are often superior to *in vitro* methods from the standpoint of sensitivity, this gap is rapidly closing due to the refinement of serological techniques and the development of quantitative methods for the study of serological reactions. Biological methods are limited by the confusing variations in individual animals so inherent in all biological systems so that it is usually necessary to test a large number of animals and to use extreme caution in generalizing on the basis of a few observations whether they be in the laboratory or in the clinic.

Neutralization. When antibodies are engendered against certain protein toxins such as diphtheria toxin, tetanus toxin, snake venoms, etc., their activity can be studied not only by the classical serological methods such as precipitation, but also by their ability to inhibit (neutralize) the toxic activity of the antigen. In some toxins, such as diphtheria toxin, the toxic part of the molecule plays a relatively minor role in the antigen-antibody reaction and the neutralizing antibodies are produced just as well with a detoxified antigen (toxoid) as with the native toxin. Since toxoids react equally as well as toxins with the antitoxic serum, serological methods such as the precipitin reaction can give no idea of toxic potency but are useful only in standardizing the antibody content of the antitoxic serum or the total antigen content of toxin preparations, which usually contain varying amounts of toxoid. The toxin-antitoxin reaction is in most instances easily reversed with complete restoration of toxic activity upon removal of antibody. As shown in Fig. 2 the extent of neutralization of toxin by antitoxin is not proportional to the amount of antitoxin added so that the kinetics of toxin-antitoxin neutralization must be based, as in the precipitin

reaction, upon the assumption that antigen and antibody combine in varying proportions depending upon the relative concentration of each, the number of combining sites, and the degree of dissociation. The molecular ratio of antibody to antigen required to give complete neutralization has been found by Pappenheimer and Robinson (111) to be 1.5-2.0 for diphtheria toxin and antitoxin.

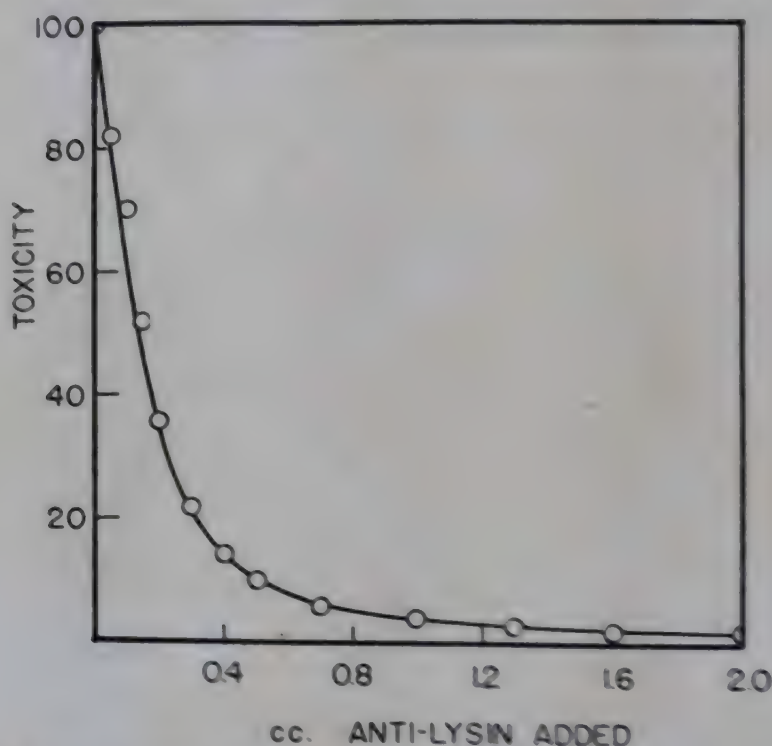


FIG. 2. The neutralization of tetanospasmin by anti-tetanus serum. Residual hemolytic activity (toxicity) as a function of the amount of anti-lysin added. Data from Arrhenius (4).

assuming a molecular weight of 184,000 for antitoxin and 70,000 for toxin. The relative excess of antitoxin required for complete neutralization may be a reflection of the dissociation of the anti-toxin-toxin complex but it would seem more likely that it indicates the necessity of masking most of the surface of the toxin molecule by antibody molecules. Tests for neutralization are usually dependent upon the sensitivity of the biological test used for the estimation of toxicity, but the test of choice will depend upon the toxin under consideration. For example, the most sensitive method for the detection of the toxicity of diphtheria toxin is the skin test, while some toxins, which owe their toxicity to the hemolysis of red blood cells, can best be titrated by *in vitro* tests.

The term neutralization has also been applied to the inhibiting effect of immune serums on the activity of viruses. In the same

sense the term might be broadened still further to include the inhibitory action of antibody on the chemical and biological activity of any antigen or infectious agent.

Hypersensitivity. The term hypersensitivity may be used to encompass all manifestations of a specifically altered physiological reaction capacity to chemical or physical stimuli. In some instances the involvement of antibody in the reactions has not been clearly established but in types of hypersensitivity such as anaphylaxis in many of the common laboratory animals or many of the classical allergies in humans there is no doubt of the participation of the antibodies. In fact, anaphylaxis offers the simplest and most sensitive method for testing the antigenicity of a substance. The parenteral injection of only a few milligrams of a functional antigen will induce an anaphylactic state in guinea pigs which is detectible after 7-10 days and which often persists for years. The tests for hypersensitivity may be made by skin tests, by intravenous injection of homologous antigen, which produces gross anaphylaxis as evidenced by severe convulsions and usually death within a few seconds, or by addition of antigen to isolated smooth muscle strips from sensitized animals. The last, which is referred to as the Schultz-Dale test or *in vitro* anaphylaxis, is superior to gross anaphylaxis for many fundamental studies since a relatively large number of tests can be performed on tissue from a single animal and only small amounts of antigen (a few micrograms) are required for the reaction.

The amount of antigen required to sensitize an animal is much smaller than that required for shocking. The lower limit for the guinea pig, which is by far the most susceptible animal, is of the order of 10^{-7} g. for sensitization with a good antigen such as serum protein or ovalbumin and 10^{-3} g. for shocking. These limits are still smaller when *in vitro* anaphylaxis is carried out as described by Nicoll and Campbell (105).

Ordinarily guinea pigs become sensitized without their serum showing any appreciable precipitin titer, but the presence of circulating antibody is easily demonstrated by the transfer of a few milliliters of serum from a sensitized animal to a normal animal. After an incubation period of a few hours an injection of homologous antigen will result in the same shock symptoms as would be obtained in the sensitized donor. The amount of antibody which is necessary for the passive sensitization of a guinea pig against ovalbumin has been found by Kabat and coworkers (75, 76) to be 0.1-0.2 mg. of either guinea pig or rabbit antiovalbumin antibody.

Small amounts of antigen are often better than larger amounts

for the production of a good hypersensitive state in guinea pigs. Hence, it is usually advisable to inject varying amounts when testing the antigenicity of an unknown substance. For example, amounts of 100 mg. or more of some azo-proteins fail to sensitize while 1-10 mg. usually do. On the other hand, preparations such as protein hydrolysates which are being tested for residual antigenicity should be used in relatively large amounts since the concentration of intact antigenic protein would be expected to be very small. The antigen/antibody ratio is probably as important in hypersensitive reactions as in any other immunological reactions for when all of the available antibody has combined with antigen the animal is "desensitized" and addition of more antigen will obviously produce no reaction until the excess of antibody is restored either by its liberation from antigen-antibody complexes or by its continued production by the animal. This same effect of excess antigen is probably responsible for the long period required for sensitization with those antigens which are only slowly eliminated from the body or which have been injected in excessive amounts.

While the antibodies responsible for the experimental hypersensitiveness of animals are the same as those which bring about the usual serological reactions, the antibodies (reagins) responsible for allergic hypersensitiveness in humans appear to be different. Thus, serum from an allergic individual will passively sensitize a normal individual but will give none of the classical serological reactions such as precipitation. In a preliminary report Miller and Campbell (97) showed that although the reagin present in the serum from individuals sensitive to egg white would not give a precipitin reaction when mixed with ovalbumin, it apparently combined with the ovalbumin to form a soluble complex and could be detected in a precipitating system of ovalbumin and rabbit antiovalbumin. The tentative conclusion was that the antibodies responsible for human hypersensitivities are probably the result of a distorted antibody-forming mechanism which produces either univalent or "weak" antibody molecules. This would explain why a mixture of reagin and antigen will not confer passive sensitization but will produce a reaction when injected into sensitized tissue. The quantitative difference in the valency of the two reagents would require only a small amount of the multivalent antigen to neutralize the sensitizing activity of univalent antibody and to give a neutral mixture with respect to antibody. On the other hand, a great excess of univalent antibody would be required to combine with all the reactive sites of the antigen in order to give a neutral mixture with respect to antigenic activity.

Immunity. In some instances the presence of circulating antibodies can only be detected by the immunity which they confer upon normal animals. This is especially true for the antibodies produced against viruses. The difficulty in detecting antibodies in such instances by serological methods is probably due to the lack of a good test antigen as well as to an antibody concentration which is not great enough to give a visible test tube reaction but which will neutralize or inhibit the activity of infective amounts of viruses. In other instances protection tests must be used for the detection of antibodies because the animal under study invariably produces low-reacting serum. For example, it is easy to immunize rats against a variety of infectious agents, particularly parasitic helminths, but specific precipitation is very difficult to demonstrate although the serum may have a very high protection titer. There is also the possibility that good serological reactions may be obtained but that the antibodies which are detected by serological tests have little or no significance in immunity.

Thus Campbell (19) found that immunization against the larval trematodes *Cysticercus crassicolis* in rats or *C. pisiformis* in rabbits resulted in the formation of antibodies which reacted with a specific polysaccharide antigen from the respective parasites, but that the removal of such antibody had no effect upon protective titers. Many such conditions must exist, where an antigenic component of an infectious agent produces a good precipitin titer, but the antibody is relatively unimportant in defense mechanisms.

III. ESTIMATION OF ANTIBODY CONTENT OF A SERUM

Having discussed briefly the methods by which antibody may be detected, we now turn to the methods for the estimation of antibody concentration. The first investigators who desired to compare the reactions of a given antiserum with a variety of antigens, as in the study of evolutionary relationships, or the reactions of a given antigen with a variety of antisera, had recourse to either of two simple methods, each of which involved the determination of the *limiting dilution* of one reagent which gave a visible *in vitro* reaction with a given amount of the other. Since precipitating sera do not give visible reactions in high dilution, more frequently a constant amount of serum was mixed, in separate test tubes, with progressive dilutions (usually two-fold dilutions) of the antigen. The determination of the limiting dilution of antigen could also be made by observing the reaction at antiserum-antigen interfaces.

It was soon shown, however, that the limiting dilution of antigen

was not a reliable index of the strength of an antiserum, since this end-point was almost independent of the antibody concentration as measured by more reliable methods, the limiting dilution of antigen often remaining constant as the antiserum was diluted several-fold (31, 32, 94). A more suitable basis for antibody estimation was provided by Dean and Webb (34). Working with horse serum as antigen and rabbit anti-horse serum antibody, they prepared various dilutions of the antibody and added to constant amounts of each antibody dilution a series of dilutions of the antigen; and they noted in each series at constant antibody the mixture in which particles of precipitate were first discernible by the naked eye. The ratio of antigen to antibody in this mixture, expressed in terms of the relative amounts of the *stock* solutions of antigen and antibody which were contained in the mixture, was referred to as the *optimal ratio*. For example, suppose that a series of antigen dilutions were mixed with equal volumes of 1:20 antiserum and that the mixture receiving 1:320 antigen was the first to particulate (flocculate). In this case the optimal ratio would be 1:16 (1:320/1:20). (The optimal proportions point usually occurs in the region of maximum precipitation (Fig. 1).) Flocculation was slower in the other mixtures and greatly retarded in mixtures containing the antigen and antibody in proportions widely different from the optimal ratio.

When they determined the optimal ratio in this way at several constant-antibody levels, Dean and Webb made the important discovery that this ratio was a constant (*cf.* Cromwell (31)). In other words, in a titration of the sort which we have described the optimal ratio of antigen to antibody was independent of the absolute concentration of these two reagents over wide limits. This observation has been verified for a number of different systems and is now a useful working generalization. A titration of this sort is now referred to as a *constant antibody optimal proportions titration*.

This titration provides a basis for the comparison of the strength of various antisera to the same antigen. Suppose that we wish to compare two antisera to the same antigen. We carry out optimal proportions titrations with a dilution, for example of 1:5, of each antiserum, using known dilutions of the antigen, and obtain values of, say, 1:8 (1:40/1:5) and 1:16 (1:80/1:5) for the optimal ratios. Since we know that the serum which is characterized by an optimal ratio of 1:8 will, if diluted 1:10, flocculate optimally with half as much antigen, and since we have found that the second serum flocculates optimally with this quantity of antigen, we conclude

that the first serum is stronger than the second, and moreover that it is approximately twice as strong, other things being equal. It is apparent, therefore, that the higher the antibody concentration, the more antigen will be required to give an optimal proportions point.

But other things are not always equal. In another section it is shown that the precipitating properties of an antiserum are determined not only by the concentration of antibody but also by its heterogeneity. Accordingly, the serums of different animals, or of the same animal at different times, may be compared only roughly by the optimal proportions method. Nonetheless the method is extremely useful in following the change in antibody titer during a course of immunization, and in other ways. It is especially useful if it is applied to the determination of an antigen in an unknown mixture of unrelated antigens (133), with the aid of a standard antiserum, or to control the purification of an antigen from a mixture. The latter operation is possible since an antiserum prepared against a mixture of antigens such as egg white will in general give several zones of precipitation when titrated with serial dilutions of the antigen mixture. Unless two zones happen to coincide, each zone corresponds to one of the antigens in the mixture. Whenever a "pure" antigen gives more than one zone with an antiserum against a source mixture, the purity of the antigen may be questioned. The sensitivity of this method for the estimation of the antibody content of a serum is limited by the ability to distinguish between the reactions occurring in the several antigen dilutions around the optimal proportions point. This may be facilitated by diluting both reagents but even then one cannot usually distinguish less than two-fold antigen dilutions.

Dean and Webb further noted that the supernatant obtained from the mixture of antigen and antibody at optimal proportions was essentially devoid of either reagent. While this is not always true of other systems, it is generally true that the antigen is completely, or almost completely, precipitated from such a mixture. We have here a simple basis for the absolute determination of the (precipitable) antibody content of an anti-protein serum. If we analyze the optimal proportions precipitate for total protein and deduct the known amount of antigen, the remainder represents the antibody (see references (31, 32)).

The quantitative determination of the absolute amount of precipitable antibody of an antiserum has been extensively developed by Heidelberger and his coworkers and others. Heidelberger and

Kendall (56, 57) have shown that, when antigen is added to an excess of antibody, for many systems the relation between antigen and antibody in the precipitate may be expressed by the equation:

$$\frac{y}{x} = 2R - \frac{R^2x}{A} \quad (\text{I})$$

where y = mg. antibody precipitated, x = mg. antigen added, A = total antibody present, R = weight ratio of antibody to antigen at the equivalence point.

The *equivalence point* is the point in an antigen-antibody titration at which neither antigen nor antibody (or only small quantities of either) is demonstrable in the supernatant fluid, and thus corresponds approximately to the point of optimal proportions. Often the equivalence point is replaced by a *zone* of antigen-antibody mixtures in the supernatants of which each reagent is absent or present only in minimal quantities. In such systems the equivalence point is taken as the midpoint of the equivalence zone. Frequently, better agreement of theory and experiment is obtained if R is determined at the antigen-excess end of the equivalence zone.

A plot of y/x against x is linear and has an intercept $2R$ and a slope $-R^2/A$. It follows that, if y and x are determined experimentally for two different mixtures of antigen and antibody in antibody excess and at constant antibody, R and A can be evaluated graphically. It must be emphasized again that A represents only the specifically precipitable antibody of a serum.

Equation I was derived with the aid of the mass law on the assumptions that antibody, known to be heterogeneous, behaves statistically as a single substance; that the reactants are multivalent with respect to each other; that the reaction occurs in steps in a series of bimolecular reactions which take place before precipitation begins, and that dissociation of the antigen-antibody compounds formed at various levels of reaction is negligible.¹

The application of Equation I to an experimental situation is illustrated in Fig. 1. Curve I is a plot of the amount of precipitate as a function of the amount of antigen added to a constant amount of antibody (7). The antigen was a five-times recrystallized preparation of hen ovalbumin (Ea) which was finally electro-dialyzed and dried from the frozen state *in vacuo*. The antiserum was a frozen-dried rabbit antiovalbumin pool which was reconstituted as a 2%

¹ A recent derivation of this equation with more suitable assumptions is given by Kendall (77).

solution in 1% NaCl. To 3 cc. of each of several suitable solutions of Ea in 1% NaCl was added 2 cc. of the 2% serum. The mixtures were allowed to stand for four hours at room temperature and 20 hours in the refrigerator. Aliquots of the precipitates were washed twice with ice-cold 1% NaCl and the precipitate nitrogen was determined by direct nesslerization. Values for *total* protein were obtained by multiplying the nitrogen values by 6.25 and are expressed for the original 5 cc. sample. Values for the amount of antigen *added* to the mixture were calculated from Kjeldahl nitrogen values on the assumption that Ea contains 15.5% N.

Note that the curve (I) appears to extrapolate to the origin, suggesting the absence of a true antibody-excess inhibition. As an increasing amount of antigen is added in a constant volume to the fixed amount of antibody the amount of precipitate increases to a maximum, and then declines as more antigen is added. In extreme antigen excess the amount of precipitate falls off to zero. It should be emphasized that each point on this curve represents the amount of precipitate which formed when the corresponding amount of antigen was added in a single dose to the antiserum.

Assuming that in the region of antibody excess (to the left of the optimum) all of the antigen was precipitated, the amount of antibody in the precipitate (y) may be calculated by deducting the amount of antigen added (x) from the total precipitate. When y/x is plotted against x , it is possible to represent the data more or less satisfactorily by a straight line, Curve II, having the equation:

$$\frac{y}{x} = 14.9 - 38x. \quad (II)$$

R is accordingly $14.9/2$, or 7.45; and A is $(7.45)^2/38$, or 1.46 mg., which is in excellent agreement with the value 1.50 mg. for the maximum antibody precipitated according to Curve I. The weight ratio of antibody to antigen in the precipitate at the graphic optimum (Curve I) is $1.50/.174$, or 8.6, which is only in fair agreement with the value 7.45. The latter is in better agreement with a value for R determined in slight antigen excess.

For a further discussion of the quantitative estimation of antibody and its application to various immunochemical problems, reference is made to Kabat's excellent review (73). Regardless of the method used certain fundamental limitations are inherent in most of the antibody estimations so far proposed. Under certain conditions it is possible to obtain a fair idea of the amount of strong precipitating antibody but this may or may not represent all the

circulating antibody, since it is well known (see below) that antisera may be extremely heterogeneous and there may be present enough weak or "univalent" antibody (*e.g.*, Rh "blocking" antibodies) to inhibit the reaction of antigen with strong antibody. It is quite evident that the time has come to develop a quantitative method for the estimation of the antibody content of a serum based on the primary antigen-antibody reaction. Such a method would ideally not be subject to the theoretical complications which characterize the present methods, which at best provide only minimal values for the total antibody of a serum.

IV. PURIFICATION OF ANTIBODIES

Methods for the purification of antibodies are based either on chemical fractionation of a serum or plasma or on the isolation of antibody from specific antigen-antibody complexes. The first method, which is usually referred to as non-specific, is applicable to the large scale commercial production of fairly pure antibody preparations; and the second method, which is referred to as specific, while obviously not applicable to large scale preparation, is of great importance for obtaining very pure preparations for experimental studies.

1. Non-Specific Methods

Since antibodies in the blood invariably appear in the plasma globulin fraction, some purification can be effected by separation of the globulins from the other serum components. This can be accomplished in the usual way by precipitation with salt or under carefully controlled conditions with alcohol (Cohn, *et al.* (29)). If the blood has not been allowed to clot, the fibrinogen can be coagulated by the addition of 5% phenol or Ca^{++} and removed. The globulins can then be precipitated by the introduction of an equal volume of saturated ammonium sulfate solution slowly through a small capillary tube with constant stirring or by dialysis against an equal volume of the saturated salt solution. The precipitation is carried out slowly in order to minimize the occlusion of undesired protein material in the precipitate. Further purification can easily be accomplished by separation of the globulin into water-soluble (pseudoglobulin) and water-insoluble (cuglobulin) fractions merely by dialysis against distilled water.

An example of such a procedure is that used by Pappenheimer, *et al.* (110) for the purification of diphtheria antitoxin from the horse in which the antitoxin occurs in the pseudoglobulin fraction.

From 900 ml. of one plasma which contained 1440 antitoxin units per ml. they obtained a pseudoglobulin preparation which represented 61.5% of the original plasma antitoxin; 32.5% of the total nitrogen of this preparation precipitated specifically with toxin. Assuming that the concentration of total protein in the starting material was 7–8% and that one gram of antitoxin is equivalent to 86,000 units of antitoxin, one may calculate that the specific fraction of the total protein was approximately doubled. In all such procedures the degree of purification will obviously depend upon the relative amount of non-antibody protein in the globulin fraction in question. At times it is advantageous to control conditions such as pH, temperature, salt concentration, etc. Instead of being treated with ammonium sulfate solution, serum is often first diluted with one volume of water and enough solid salt is then added to give a final concentration of 220–230 g. per liter. The mixture is allowed to stand at room temperature or slightly above for 12–18 hours and then filtered. The precipitate is dissolved and reprecipitated several times, depending upon the degree of purity desired. The pseudoglobulins are then removed partly by dialysis and partly by isoelectric precipitation. The salt-precipitated globulins are redissolved in water and dialyzed again until a faint turbidity develops or the salt concentration is less than 0.3%. The mixture is then adjusted to about pH 5.6 (with CO₂, potassium acid phthalate, or acetic acid) and the precipitate of pseudoglobulin removed.

The purification of horse antipneumococcus antibody is unique in that this protein appears in the euglobulin fraction, which often contains very little non-antibody protein. Concentration of antibody can be effected by removal of electrolytes, salt precipitation, or alcohol precipitation. Felton's method (46) is one of purification with 15–20% ethanol at 0° and consists in adding 95% ethanol to undiluted horse antiserum, the temperature being kept at 0° during all of the operations. The final yields of antibody are of the order of 80% of the original serum antibody, and 80% of the final product can be accounted for as antibody. The precipitation of Rh antibody has recently been reported by Witebsky, *et al.* (149) to occur on dialysis against distilled water. It would seem that the Rh agglutinating antibody is a euglobulin and the Rh "blocking" antibody a pseudoglobulin.

The possibility of using proteolytic enzymes for the purification of antibodies has been widely explored and is apparently of value only in the preparation of horse antitoxins. The method consists essentially in diluting antitoxin serum with one volume of water

and digesting with an "excess" of pepsin at pH 4.0 and 37° for about 24 hours. The resulting solution is then treated with calcium phosphate to remove the residual pepsin and dialyzed free of salt. The soluble protein is then precipitated with ammonium sulfate, redissolved and redialyzed against physiological saline. The final product may be heated to 58° for a short time to remove more of the inert protein. The final antibody product has a very low functional antigenicity and contains about 80% of the activity of the original serum.

Other non-specific methods which may be important but are certainly not practical at the present time consist of fractional centrifugation (especially applicable to the "heavy" antipneumococcus component of horse serum) and electrophoresis.

2. Specific Methods

The possibility of separating antigens and antibodies from an insoluble precipitate of the two is suggested by the observation that various treatments tend to inhibit precipitation or to produce resolution of a precipitate. For example, the specific antigen-antibody reaction is influenced by temperature, H-ion concentration, and simple haptens. Since the antigen-antibody reaction may be considered to be reversible then the imposition of these inhibiting factors may be expected to produce considerable separation of the two components; and, if they differ sufficiently in their properties, one or the other may be isolated by precipitation, absorption, or electrophoresis, or if the antigen is in an insoluble form such as an intact cell, merely by dissociation and centrifugation. The following procedures describe the general methods for the recovery of antibody from insoluble and soluble antigens.

The use of an insoluble antigen offers the advantage that it can be removed from the system at any time by centrifugation. However, it is necessary that conditions be controlled to assure that no constituents of the antigen dissolve, since some of the difficulties experienced in isolation of antibodies from bacterial cells have been due to dissolution of antigenic constituents of the cell, particularly when strong acids or alkalis have been used for dissociation. One must also consider that a complex antigenic structure such as a bacterial cell usually contains many different antigenic components each of which may absorb its respective antibody. The resulting preparation may consist entirely of antibody protein but still show all the vagarity and cross reactions of the original serum.

Pneumococcus is unique in that the cell surface is fairly homoge-

neous antigenically and that aside from the type-specific polysaccharide one need consider only the cross-reacting C-substance. The bacteria are usually first killed by heat, alcohol, formalin, or a combination of heat (70–80° for 30 minutes) and formalin (1.0%), as suggested for pneumococcus by Goodner, Horsfall and Dubos (51), and are then carefully washed to remove all soluble antigens. A dense suspension of the washed organisms is then added to the antiserum, which is often diluted with 1–2 volumes of 0.9% saline if the titer is high. Sufficient antigen should be added to remove essentially all the antibody from solution; the completeness of the removal is determined by testing a sample of the absorbed supernatant solution either with more of the absorbing antigen or, in the case of pneumococcus, with the soluble type-specific polysaccharide. The mixture of antigen and antiserum is usually kept at 0° for 24–48 hours, and is then centrifuged and washed in the cold until washings are free of protein. Removal of antibody from the antigen is then accomplished by several methods. One of the simplest and perhaps first to be tried is the specific absorption of antibody by cells at 0° and resuspension at 40–60°. At this higher temperature some dissociation of the cell-antibody complex takes place and the antibody can be recovered in the supernatant solution after removal of cells by centrifugation. By such a procedure Landsteiner (78) was able to obtain some antibody from red blood cells and a variety of bacteria.

Jenkins (68) has recently reported the recovery of antibody from *B. typhosus* and *B. paratyphosus* A and B by absorption at 0° and dissociation at 56°. Unfortunately too little quantitative data are available to give much idea of the yields or purity of antibody so obtained. Other methods which have been widely studied involve the use of dilute acids, bases, or strong salt solutions. The only quantitative investigations on the isolation of antibody from cells have been reported by Heidelberger and Kabat (55) and Lee and Wu (90), both investigations being carried out with pneumococcus.

Heidelberger and Kabat used essentially three methods for dissociation involving resuspension in 1) 0.9% NaCl solution at elevated temperature, 2) 15% NaCl solution, and 3) 10% Ba(OH)₂ solution. The purity and yields obtained with antiserum from several sources and having different titers showed a great variation. Two preparations obtained by resuspension of the agglutinated bacteria in 0.9% NaCl at 37° gave yields of 1.4 and 0.7% with purities $\left(\frac{\text{antibody N}}{\text{total N}} \right)$ of 46 and 34%, respectively. When the

organisms were suspended in 15% NaCl the yields were 3.7–31% and the purities 20–92%. The best yields were obtained when organisms were suspended in saturated $\text{Ba}(\text{OH})_2$ solution plus 10% BaCl_2 solution. When these supernatants were freed of barium ion by dialysis yields of antibody varied from about 3.4–45% with purities of 71–97%. The latter yields would probably have been much greater except that in each instance the cell-antibody complex had been first extracted with 15% saline. It would seem that the latter method using barium was the one of choice.

Lee and Wu found that too much antigenic material was dissolved from the pneumococcus cell in dilute sodium hydroxide solutions (above pH 8.0) to afford good preparations of antibodies, hence they concentrated on the dissociation of antibodies by acids. By suspending agglutinated pneumococci in dilute HCl solutions for 10 minutes they found that upon removal of the organisms and neutralization of the solution, about 87% of the absorbed antibody was removed at pH 2.03 and 49% at pH 4.08. The purity of the preparation was calculated to be approximately 95%. The absorption and dissociation of antibody from insoluble antigenic preparations other than cells has been studied by a variety of workers. For example, Landsteiner and van der Scheer (86) used azo-stromata to absorb rabbit antibodies against several different haptens and then dissociated the complex with dilute acetic acid. They were able to obtain about 50% of the original serum antibody in a fair state of purity. Meyer and Pic (96) adsorbed Besredka's antigen on kaolin and after allowing the kaolin-antigen to react with antiserum at 0° were able to dissociate a small amount of antibody at 60°.

We have investigated the possibility of isolating antibodies against ovalbumin by treating rabbit antiserum with a finely divided suspension of heat-coagulated ovalbumin or of surface-denatured ovalbumin. The mixture of denatured ovalbumin and antiserum was allowed to react for 24 hours at 0°; then the particles were removed, washed, and finally resuspended in 0.9% NaCl solution, and the mixture was adjusted to pH 3.2. The mixture was stirred for 30 minutes at room temperature; then the insoluble residue was removed and the dissociated antibody protein was precipitated with ammonium sulfate and dialyzed against 0.9% NaCl for 24 hours. The antibody recovered was approximately 10% of the amount present in the original antiserum and approximately 100% of the amount absorbed from the serum.

Although the use of insoluble antigens has certain obvious advantages it is apparent that soluble antigens may be more efficient,

because of their extensive surface, for absorbing antibodies from serum. Studies with soluble antigens have been largely concerned with precipitates of pneumococcus specific polysaccharides and their respective antibodies. Thus, Felton (47) reported the isolation of horse anti-SI and anti-SII from specific precipitates by dissociation with strontium or calcium phosphate at pH 9.0–9.6. Although these preparations contained 70–80% antibody as judged by precipitation tests, the lack of quantitative data does not permit careful estimation of yields.

The first quantitative treatment of antibody purification was described by Heidelberger and Kendall (58) and Heidelberger and Kabat (55), who found that strong sodium chloride (15%) solutions would alter the polysaccharide-antibody ratio in the precipitate in such a manner as to liberate antibody. Thus, precipitates were obtained by adding specific polysaccharide to antiserums from horse, rabbit, sheep, pig, and cow. The antigen and antiserums were mixed in ratios such that there was a slight excess of antibody (*i.e.*, antibody could still be detected in the supernatants) and then allowed to stand until flocculation occurred. The precipitates were then washed with 0.9% NaCl until no heat coagulable protein was found in the supernatants and finally suspended in 15% NaCl solution for one hour at 37°. The solutions were then dialyzed against 0.9% saline plus a little toluene for a preservative until equilibrium was reached. This method gave yields which varied from 0.5 to 24% of the antibody present in the original serum and purities of 59 to 100%. When precipitates were dissolved in barium hydroxide and barium chloride solutions and then neutralized with acetic acid, antibody was obtained in the supernatants in yields which varied from 0.3 to 21% and purities of 59 to 97%. However, as in the studies with agglutinated cells, the extractions with barium solutions were made after salt extraction, hence the values are probably much lower than if the barium extraction had been made first. Liu and Wu (91) studied the dissociation of SI-horse antibody complexes by acid and base and obtained yields of 44.5% and 37.3% for preparations obtained at pH 4.25 and 10.09, respectively. They reported purities of 91% for the acid preparation and 85% for the alkali preparation.

The method of salt dissociation, which has been so successful in purification of antibodies against pneumococcus, has not consistently led to the liberation of antiovalbumin from ovalbumin-antiovalbumin precipitates (*cf.* (108)). However, we have found (unpublished) that fairly good preparations of antibody can be ob-

tained under suitable conditions by either acid or base dissociation. For example, precipitates obtained in the equivalence zone by reaction of rabbit antiovalbumin and ovalbumin were thoroughly washed with 0.9% NaCl, suspended in more of the salt solution, adjusted to pH 3.5–2.5 with HCl and allowed to stand two hours at room temperature. After the insoluble residue was centrifuged off, the supernatant protein was precipitated by half saturation with ammonium sulfate, washed, then dissolved in 0.9% NaCl and dialyzed against 0.9% NaCl at about pH 7.0 until free of sulfate. When the solutions of such preparations were made equal in volume to the original sample of antiserum and tested with ovalbumin, the optimal proportion zones were very similar to those of the original serum and, as nearly as could be determined by such methods, gave yields of 70–90%. The purity of the preparations $\left(\frac{\text{antibody N}}{\text{total N}} \right)$ was of the order of 90%. Similar extractions with dilute sodium hydroxide solutions (pH 10–11) gave lower yields of approximately 25%.

We have also investigated the purification of antibodies against azo-arsanilic acid. Of several systems which we have studied the two most promising are 1. dialysis of a specific precipitate against a simple inhibiting hapten when the precipitating antigen is small enough to pass through the dialyzing membrane and 2. acid dissociation of an antigen-antibody complex in which the antigen is acid-insoluble. During the course of investigations on the serological reactions of simple precipitating dye antigens, phenylarsonic acid dyes were found which were suitable for this work. The antigen and rabbit anti-arsanilic acid antisera were mixed in optimal proportions and allowed to react at 4° for 48 hours. The precipitate was collected by centrifuging, washed free of excess dye, and then dissolved in 10% sodium arsanilate (which functions as an inhibiting hapten); and, when the dialyzable precipitating antigen was used, the dissolved precipitate was dialyzed against successive changes of 10% arsanilate (pH 7.0–8.0) until free of color. The sodium arsanilate was then removed by dialysis against saline. Although this method has the advantage of being extremely simple it has the disadvantages of involving the use of a rather inefficient precipitating antigen and of requiring several weeks of dialysis to remove the dye.

When a larger acid-insoluble dye antigen was used, the arsanilate solution of the antigen-antibody precipitate was acidified to about

pH 3.0 and stirred for two hours at room temperature. The mixture was then centrifuged and saturated sodium chloride solution was added to the supernatant to give a definite precipitate. This precipitate, which was practically pure antibody, was removed and the remaining protein was precipitated by further addition of salt solution. The latter fraction usually contained traces of dye which could eventually be removed by repeated fractional precipitation of the antibody with saturated salt solution at pH 3.0–3.5. This method gave extremely good results and quantitative analyses on the precipitates and solutions indicated approximately 100% recovery of the original antibody of the antiserum. The resulting protein was completely precipitable by antigen at optimal proportions and was electrophoretically homogeneous. Essentially the same principle was utilized by Sumner and Kirk (132) for the purification of rabbit antibody against urease, which is quickly denatured to an insoluble condition by dilute acids. For example, they obtained a precipitate from a mixture of urease and antiurease (proportions not given), washed it several times, and resuspended it in 0.1 *N* HCl (pH not stated). After a few minutes of stirring the insoluble urease was removed. The resulting supernatant, after neutralization, gave qualitative antibody reactions which indicated that over 50% of the antibody in the original sample had been recovered.

It is becoming increasingly evident that many types of investigations of the fundamental nature of antibodies require purified preparations for interpretable results. It is important, therefore, that better methods be developed for the isolation and purification of antibodies. At the present time the foregoing methods are the most practical and of these the acid dissociation at about pH 3.0 would appear to be the method of choice, especially when the antigen is acid-insoluble.

V. FEATURES OF ANTIBODIES AND OF ANTIBODY FORMATION

1. The Chemical Nature of Antibodies

At the present time it is generally accepted that antibodies are proteins of the class of serum globulins. For early evidence of the protein nature of antibodies, derived from studies of their chemical composition, solubility, denaturability, and serological reactions, reference is made to Marrack's excellent monograph (93). The important outcome of this early work was the demonstration of an

intimate association of antibody activity with the proteins of immune plasma. Recently, additional evidence has been provided by the study of the physico-chemical behavior of purified antibody preparations, as revealed by diffusion, electrophoresis, and sedimentation. The results of these studies serve to remove the last reasonable doubts that antibody activity is inseparably associated with molecules of serum globulin. Occasional claims of the non-protein nature of antibodies characteristically fail to be supported by the isolation of sufficient quantities of material for chemical characterization; and the superior sensitivity of immunological over chemical methods for the detection of small quantities of antibody is overlooked.

2. A Comparison of the Properties of Antibodies and Other Serum Proteins

The study of the physical, chemical, and serological properties of antibodies, designed to reveal differences between antibodies and other serum proteins, as well as between various kinds of antibodies, is extremely important for a more thorough understanding of significant aspects of the structure of antibodies and of the mechanism of their formation. While the characterization of antibodies is far from complete, much progress has been made in the past decade and more may be expected with the development of new and more precise methods for the study of proteins. At present the principal lines of attack are based on studies of: 1. electrokinetic properties; 2. molecular weights; and, 3. antigenic specificity. The discussion of the elementary and amino acid composition of antibodies, a subject of obvious bearing on the question, has been omitted here because of lack of sufficient data (see Marrack (93)). Likewise, the solubility properties of antibodies are not discussed since no useful correlation has been established.

Perhaps the most fruitful information has been obtained from studies of the electrical charge of plasma proteins in the presence of approximately physiological concentrations of H-ions and electrolytes. Thus, it is now generally recognized from electrophoresis data that antibodies are usually associated with only one of the five major electrophoretically distinct plasma proteins, namely, the *gamma* globulin component. Generally, the *gamma* globulin is considerably increased during immunization. If an immune serum is absorbed with homologous antigen so as to remove the antibody, concomitantly the quantity of *gamma* globulin, as shown by the area under its peak in an electrophoretic diagram, is reduced; in-

deed, this reduction in area consequent to specific absorption may be taken as an approximate measure of the antibody content of the unabsorbed serum.

In rabbit antiserums the antibody characteristically migrates with the *gamma* globulin. In rare cases (141) there may occur an extra-normal electrophoretic component, closely associated with the *gamma* globulin and resembling the T component observed in certain horse antiserums (see below). It has not been shown whether this extra-normal component bears the specific antibody activity.

The situation is considerably more complex, however, when one is considering antiserums developed in the horse. Van der Scheer, *et al.* (142) separated horse antiserums experimentally into three distinct classes, *viz.*: 1. those in which the *gamma* globulin was increased; 2. those in which a new electrophoretic component, T, was present along with the normal quantity of *gamma* globulin (*e.g.*, tetanus antitoxin); and, 3. those in which the T component was present along with an enhanced *gamma* globulin (*e.g.*, diphtheria antitoxin).

Pappenheimer, *et al.* (110) purified a pseudoglobulin with diphtheria antitoxic activity from horse serum by ammonium sulfate precipitation and dialysis which was homogeneous by sedimentation, diffusion, and electrophoresis but which was distinctly different from normal *gamma* globulin by the latter criterion. This difference could have been the result of treatment during purification. However, only 43.5% of the purified pseudoglobulin was specifically precipitable as antitoxin, so that approximately half the material consisted of inactive material which was otherwise identical with the antitoxin. There remains the possibility that the fraction which was inactive by specific precipitation would have been found active in a biological protection test.

Tiselius (134) studied the electrophoretic behavior of purified horse and rabbit antibodies against pneumococcus polysaccharide and found them to have mobilities which differed from any which are found in normal serum. Tiselius and Kabat (135) also found some evidence that horse antipneumococcus serum contained a component which migrated between the *beta* and *gamma* globulins and which was entirely removed by absorption of the antiserum with antigen. Other serums which they studied gave no clear evidence of any distinct antibody boundary. Upon continued immunization of horses they found that two more extra-normal components appeared. In contrast to these findings, Moore, *et al.* (99)

were unable to obtain any separation of an antibody component from the *gamma* globulin even by prolonged electrophoresis at various H-ion concentrations. Only in one instance (with a Felton concentrate) did they observe an extra-normal component.

Studies of the molecular weights of antibodies have given more complex data since it has been generally found that antibodies may fall into two general molecular weight classes, namely, 160,000 and 1,000,000. The anti-S antibodies of horses, pigs, and cows usually appear in the "heavy" (1,000,000 m.w.) component (72), which is usually increased during immunization and at times may contain all the anti-S antibody in a high state of purity. However, upon prolonged immunization anti-S antibodies often appear in the "light" (160,000 m.w.) component, and Fell, *et al.* (45) reported anti-S horse serums in which most of the antibody was in the light component. The antitoxin antibodies of horses always appear in the light component. Antibodies in man and rabbits have generally been associated with the light component regardless of the kind of antibody. However, Deutsch, *et al.* (35) have recently reported some ultracentrifuge studies of *gamma* globulins (γ_1 and γ_2) from human serum which indicated that the heavy component (γ_2 with a sedimentation of 18–20 svedbergs) contained a high concentration of various antibodies.

The third line of attack, namely, comparison of the antigenic structure of antibodies and normal globulin, would be expected to reveal relatively fine differences in the molecular structures of different proteins. However, the weight of evidence indicates no antigenic difference between antibody and normal globulin of the same animal; in other words, the antibody combining site makes no appreciable contribution to the antigenic specificity of the antibody molecule. Thus, Landsteiner and Prašek (81) found that antibodies formed against normal horse serum reacted equally well with normal horse serum and horse anti-typhoid serum. Wright (150) reported that the multiple optimal proportions zones obtained with rabbit anti-horse serum and normal horse serum correspond to those obtained with horse antipneumococcal serum as the test antigen. More quantitative studies by Treffers and Heidelberger (138) and Treffers, Moore, and Heidelberger (139) also failed to detect any antigenic differences between comparable normal and antipneumococcal horse globulins, although some differences were found when the low molecular weight antitoxic globulin, or the corresponding normal globulin, was compared with the high molecular

weight antipneumococcal globulin, thus confirming the earlier observations of Ando, Takeda, and Hamano (2).

All of these studies have the common feature that the antigenicity of an antibody was compared with the antigenicity of a selected normal globulin. Taking a different approach, one of us (D. H. C.—unpublished) carried out experiments to determine whether homologous antibodies prepared in animals of different species had any antigenicity in common. Guinea pigs were sensitized against horse anti-Type I (pneumococcal) globulin contained in the specific precipitate with the homologous polysaccharide and were later tested for anaphylaxis with a preparation of Lederle's concentrated and partially purified rabbit anti-Type I serum. Neither gross anaphylaxis nor *in vitro* Schultz-Dale reactions were observed in any instance. Thus, the proteins had a common antibody functional activity but no common antigenicity.

These negative findings may be explained in several ways. If, as is sometimes postulated, antibody specificity is based on a particular folding of the polypeptide chains which make up the molecules of protein, it is possible that such a secondary configuration is not stable with respect to the degradative processes incident to antigenicity.

Furthermore, to the extent that the antibody combining site consists of a confined cavity rather than a prominence, it is likely, according to several theories of antibody formation, that an anti-antibody would not reflect the structure of the combining site precisely. It may be readily conceived that an antigenic prominence will be more accessible than a confined pocket to antibody precursors of limited plasticity (67). There is some evidence that pocketness is an attribute of the antibody combining site.

There is a third, quantitative argument which contributes to the explanation of the negative results in these experiments. If we accept Neurath's calculations (101) of the absolute dimensions of protein molecules and if we assign a reasonable value to the surface area of a combining site, the proportion of the antibody surface which is active is so small that it becomes unlikely that the combining site would make significant contributions to the antigenicity of the antibody molecule, on the assumption that the combining site is not an especially powerful determinant. For example, a prolate ellipsoid with axial dimensions of 300 Å and 37 Å would have a surface area of approximately 26,000 Å². Although it is not possible accurately to evaluate the area of the combining sites of antibodies

against naturally occurring antigens, it is known that the size of the combining site specifically directed against a phenylarsonic acid group approximates the size of the group itself (116) which has a surface area of approximately 100 \AA^2 , or less than 1% of the total area.

An alternative, and apparently more rational, experimental approach to the problem of the antigenicity of the combining site appears not to have been exploited. If one considers the antibody against an artificial antigen, such as a conjugated protein, for which an inhibiting hapten is available, then theoretically if the combining site is antigenic the hapten should partially inhibit the precipitation reaction of the antibody with its anti-antibody. This follows since the combining site of the anti-antibody would bear a stereochemical resemblance to the hapten. It is true that a negative result would not constitute proof since the combining site-anti-combining site reaction may be expected to make only a small contribution to the total precipitation reaction; but the experiment, as we have outlined it, has a more adequate theoretical basis than any of the experiments which have been carried out.

It is apparent from the studies made so far that aside from electrokinetic properties no distinguishing characteristics have been found for antibody globulin. Electrokinetic properties more or less limit antibodies to a single-serum component, although this reflection of the over-all electrical charge gives little information at present as to the intrinsic structure of the antibody molecule. The fact that antibodies are so closely associated with the *gamma* globulin component has led some immunologists to believe that all *gamma* globulin represents antibody. This, of course, immediately raises the question as to what constitutes the so-called normal protein to be used as a reference material in the comparison of antibody with other proteins.

It has been common practice to consider normal globulin as one without antibody activity. This, of course, is a relative property since tests for antibody activity are usually carried out with only one antigen. The lack of antibody activity could well be due to two factors. The first, and most obvious, is that antibodies are probably present for a variety of antigens which have not been considered in tests for antibody activity. The increase in so-called normal globulin along with antibody during immunization may actually represent antibodies against antigenic components which are not considered. For example, immunization against pneumococcus is usually accomplished by injection of the entire bacterial cell while tests for

antibody are made with only the purified polysaccharide component. It is also possible that antibodies are formed against internal portions of the antigen molecule made available by degradation inside the animal body; these antibodies may not react with the surface groups of the native antigen. The second factor is that due to the heterogeneity of antibodies (see below) a considerable fraction might fail to be detected by ordinary serological reactions.

3. The Specificity of the Antibody Combining Site

The statement that serological reactions are characterized by a high degree of specificity may be considered the fundamental proposition of modern immunochemistry, although it is not to be inferred that the specificity is absolute.

If one is provided with a group of different antisera and a group of corresponding antigens, he can, by studying the reaction of each antiserum with each antigen, select pairs of one antiserum and one antigen which react with each other most strongly. Depending on the content of the two groups of reagents, he may find that one of the antisera reacts exclusively with one antigen, or *vice versa*; but if the two groups are sufficiently large, and especially if the group of antigens contains related materials (*e.g.*, serum proteins, or hemoglobins, or ovalbumins from a number of zoologically related species; or a number of different proteins from the same species) it is likely that the exclusive feature will be absent and instead a given antiserum will give graded reactions (cross-reactions) with antigens related to the homologous antigen. This general result, which Landsteiner (80) describes as "the disproportional action of a number of similar agents on a variety of related substrata," provides the phenomenological basis for the broadest meaning of specificity in immunology.

The explanation of the phenomenon of specificity constitutes one of the chief theoretical problems of immunochemistry. The most important clue to the basis of specificity has been provided by experiments on the serological reactions of chemically modified antigens. These experiments, and especially the group of experiments, initiated by Landsteiner, which deal with the serological reactions of conjugated proteins prepared by combining proteins with known organic groups, have led to the substantiation of the idea, originally introduced into immunology by Ehrlich, that specificity is based on a structural correspondence or *complementariness* between the combining regions of antibody and antigen.

According to the present notion of complementariness, the spa-

tial relation between the homologous combining regions is such as to allow many points of contact between atoms and groups of one and atoms and groups of the other, thus enabling the effective attractive interaction between bond-forming elements of both. Since antibodies can be studied as antibodies only through their combination with antigens, an important requirement, from the point of view of the chemist, is that the complementariness must be sufficiently great to allow the formation of a bond which is stable at ordinary temperatures. Structural chemistry has provided the explanation both of the formation of such a stable bond and of the specificity of the bond-forming reaction; the principles underlying this explanation have been presented by Pauling (113).

According to Pauling three types of forces may be expected to participate significantly in the attractive interaction between molecules of antigen and antibody; these are the electronic van der Waals forces, the electrostatic (coulomb) forces, and the hydrogen-bond-forming forces. The individual bonds formed by the operation of these forces are relatively weak, with bond energies ranging up to about 5 kilocalories per mole of interacting pairs of atoms or small atomic groups, and it is only through the integration of many such forces that an intermolecular bond which is stable at ordinary temperatures may be formed. The necessity for the integration of many weak attractive forces, together with the inverse relation between the magnitude of these forces and the distance between interacting atoms and groups (*e.g.*, the electronic van der Waals forces diminish with the seventh power of the separation) provide the basis for specific intermolecular interaction, since they require that the spatial pattern of the bond-forming elements of the antibody combining site match closely the spatial pattern of the bond-forming elements of the combining region of the antigen. To quote Pauling (113),

"The forces of van der Waals attraction, hydrogen-bond formation, and interaction of electrically charged groups are in themselves not specific; each atom of a molecule attracts every other atom of another molecule by van der Waals attraction, each hydrogen atom attached to an electronegative atom attracts every other electronegative atom with an unshared electron pair which comes near it, and each electrically charged group attracts every other oppositely charged group in its neighborhood. Similarly the van der Waals repulsive forces are non-specific; each atom in a molecule repels every other atom of another molecule, holding it at a distance corresponding to the sum of the pertinent van der Waals radii.

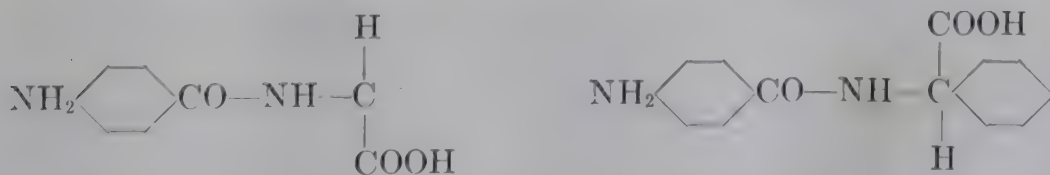
"We see, however, that specificity can arise in the interaction of

large molecules as a result of the spatial configuration of the molecules. Two large molecules may have such shapes that the surface of one cannot be brought into contact with the surface of the other except at a few isolated points. In such a case the total electronic van der Waals attraction between the two molecules would be small, because only the pairs of atoms near these few isolated points of contact would contribute appreciably to this interaction. Moreover, the distribution of positively and negatively charged groups and of hydrogen-bond forming groups of the two molecules might be such that only a small fraction of these groups could be brought into effective interaction with one another for any position and orientation of one molecule with respect to the other. The energy of attraction of these molecules would then be small.

"If, on the other hand, the two molecules possessed such mutually complementary configurations that the surface of one conformed closely to the surface of the other, there would be strong electronic van der Waals attraction between all of the atoms on the surface of one of the molecules and the juxtaposed atoms of the complementary surface of the other molecule. And if, moreover, the electrically charged groups of one molecule and those of the other were so located that oppositely charged groups were brought close together as the molecules came into conformation with one another, and if the hydrogen-bond forming groups were also so placed as to form the maximum number of hydrogen bonds, the total energy of interaction would be very great, and the two molecules would attract one another very strongly. It is clear that this strong attraction might be highly specific in the case of large molecules which could bring large areas of their surfaces into contiguity.

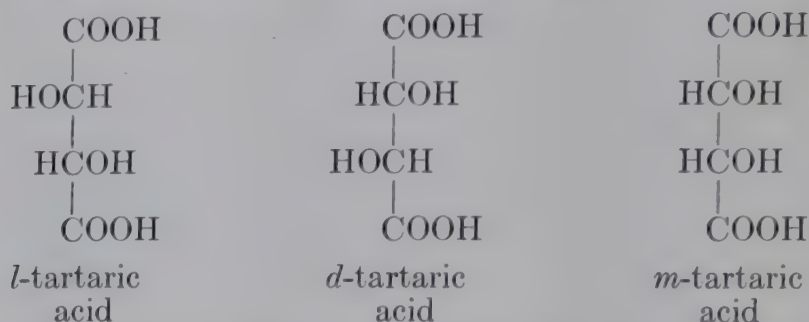
"A molecule would show strong attraction for that molecule which possessed completely complementariness in surface configuration and distribution of active electrically charged and hydrogen-bond forming groups, somewhat weaker attraction for those molecules with approximate but not complete complementariness to it, and only very weak attraction for all other molecules."

The many experiments which establish complementariness as the basis of specificity have been thoroughly discussed by Landsteiner (80), hence only a few examples will be briefly considered here. Landsteiner and van der Scheer (82) conjugated proteins with *d*- and *l*-*p*-aminobenzoyl phenylaminoacetic acids:



by means of an azo linkage. The injection of these azoproteins into rabbits resulted in the production of antisera which distinguished

the two isomeric haptens. In another similar series of experiments Landsteiner and van der Scheer (83) were able to distinguish serologically the three stereoisomers of tartaric acid:



Similarly, Avery and Goebel (5) were able to distinguish glucose and galactose by means of antisera against proteins conjugated with the *p*-aminophenol- β -glycosides of the respective simple sugars. Perhaps the upper limit of specificity was reached in a study of the serological properties of α - and β -glucosides (6). Serums against the respective glucosides (coupled to protein through a benzeneazo group) could be used to distinguish these isomers although there was considerable cross-reaction, as might be expected.

The specificity of serological reactions has found extensive application in forensic and clinical medicine. Examples of this application are the identification of the species source of blood stains, the proof of non-paternity, and the typing of serums prior to transfusion.

The apparent lack of specificity which is often encountered with complex antigens is usually a reflection of the heterogeneity of the antigen preparation. For example, crystalline ovalbumin may give two or more optimal proportions zones of precipitation as result of the presence of some partially denatured protein or other impurities. Cross reactions between apparently unrelated complex antigens are probably the result of common haptenic groups. A more detailed discussion of specificity and the heterogeneity of antibodies is included in the following section.

4. The Heterogeneity of Antibodies Engendered by a Pure Molecular Antigen

It is becoming increasingly evident from quantitative studies that the antibody molecules in an antiserum prepared against a pure molecular antigen are not homogeneous with respect to their specific reaction capacity. From the point of view of modern theories of antibody formation (see below), according to which the

combining sites of antibodies attain their definitive configurations in spatial contact with the antigen, we may expect great variety in the configuration of the combining sites, resulting from variations in the portion of the antigen surface which acts as template, in the nature of the groups (*e.g.*, amino acid residues) which finally constitute the combining site, and in the history of the structural relations among these groups before they come under the influence of the antigen. On this basis we may expect that an antiserum against

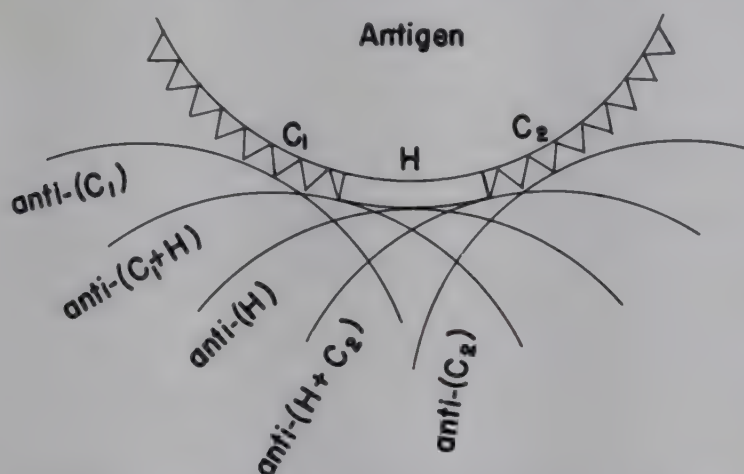


FIG. 3. Diagram representing heterogeneity with respect to homology. There is shown a portion of the surface of a conjugated protein antigen, consisting of hapten (H) and adjacent regions of the carrier (C_1 and C_2) on opposite portions of the hapten. Of the larger number of possible combining sites, which may be considered to fall in a continuous series with respect to their homology, there are shown only five, of which one (anti-H) is directed solely against the hapten, two (anti- C_1 and anti- C_2) are directed solely against the carrier, and two (anti- (C_1+H) and anti- $(H+C_2)$) are directed against adjacent portions of the carrier and hapten simultaneously.

a conjugated protein antigen would contain a spectrum of combining sites, some directed against various portions of the haptenic group, some against various portions of the carrier, and some against structural complexes involving adjacent portions of the hapten and carrier (Fig. 3); this kind of variation among the combining sites, described in terms of the portion of the antigen molecule toward which the combining sites are directed, we refer to as *heterogeneity with respect to homology*.²

² In immunology the term *homology* primarily expresses the reciprocal relationship between an antigen and the antiserum or the antibodies prepared against it. Secondly, either the antigen or the antibody is said to be *homologous* with respect to the other reagent. Thus, ovalbumin is the homologous antigen with respect to antiovalbumin, and antiovalbumin is the homologous antibody with respect to ovalbumin. The antibodies in an antiserum against a conjugated protein are said to be hapten-homologous if they are directed against the hapten or carrier-homologous if they are directed against the carrier. The term homology, applied to an antibody combining site, indicates the portion of the antigen molecule toward which the combining site is directed.

While the combining sites would not be expected to fall into sharply defined classes with respect to homology, in suitable instances it should be possible to fractionate the antiserum so as to provide antibodies with the capacity to react with one group of the antigen but not with another (see above). Moreover, we may expect that combining sites of the same homology, *i.e.*, combining sites directed against the same portion of the antigen, would vary in their *strength of combination* with the antigen, depending on the number and intensity of the individual weak forces which could be brought into play.

In the reaction between antigen and combining sites of different combining strength, the competition between the various combining sites for the combining regions of the antigen would lead under suitable conditions to the relative concentration of the strong combining sites in the precipitate and of the weak combining sites in the supernate.

We may further expect that combining sites of the same strength and homology would vary in their *kind of complementariness* to the antigen, *i.e.*, in the relative contributions of various kinds of forces to the stability of their bonds with the antigen. We could presumably detect such a variation by altering the reaction medium so as to modify the contribution of one or another kind of force; *e.g.*, salts would be expected to diminish the contribution of the electrostatic forces. Or we could study the reaction of combining sites of the same strength and homology with a number of substances related structurally to the antigen but deviating from it in different known ways. These substances would differ in their strength of combination with the various combining sites and the difference could ideally be interpreted in terms of differences in the kind of complementariness of the combining sites.

The combining sites, differing from one another in the possible ways described above, would occur singly or in groups on the various antibody molecules of the antiserum. *A priori*, we must allow considerable randomness in the kinds of combining sites that occupy the same antibody molecule; this means that two combining sites on the same molecule may differ independently of each other with respect to homology, combining strength, or kind of complementariness.

To summarize, the explanation of serological specificity in terms of a complementary structural relationship between the combining regions of antigen and antibody, together with the template theory of antibody formation, lead to the expectation of the following

reasonable sorts of heterogeneity in an antiserum prepared by the injection of a pure, molecular antigen:

1. Heterogeneity with respect to valence: The antibody molecules of an antiserum may differ in the number of combining sites which each contains.

2. Heterogeneity with respect to homology: Combining sites may vary with respect to the portion of the antigen surface toward which they are directed.

3. Heterogeneity with respect to combining strength: The individual combining sites may vary in their strength of combination with the antigen.

4. Heterogeneity with respect to kind of complementarity: The individual combining sites may form bonds with antigen which differ in the relative contributions of van der Waals forces, electrostatic forces, or hydrogen-bond-forming forces to their stability.

Only with respect to valence are we justified in expecting distinct categories of antibodies; and even here we may encounter combining sites which have the capacity to react with more than one antigen molecule simultaneously. The other sorts of heterogeneity are conceivably less discontinuous, but it may be possible to fractionate an antiserum according to one or another criterion so as to separate antibodies which differ in their behavior. An antibody fraction which was homogeneous by one criterion could reasonably be heterogeneous by other criteria.

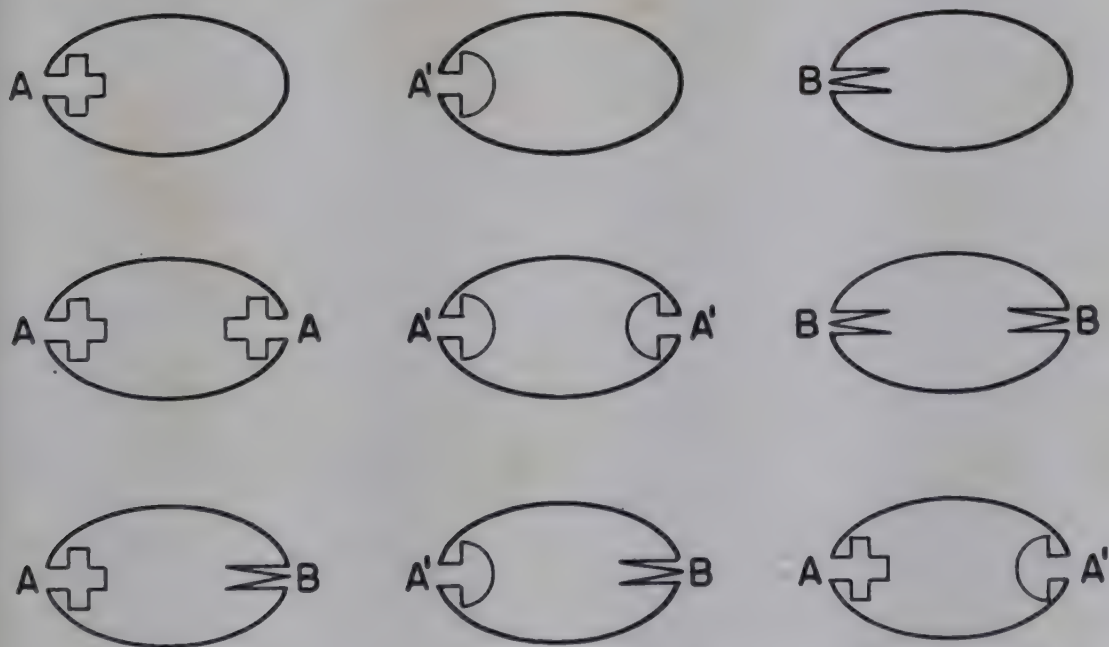


FIG. 4. Diagrams representing possible antibody molecules in a simple antiserum, illustrating heterogeneity with respect to valence, homology, and combining strength. (Simplified, see text.)

Figure 4 serves to illustrate diagrammatically some of the possible kinds of heterogeneity in an antiserum. The example is taken of a serum prepared by the injection of a homogeneous, molecularly dispersed protein antigen. The heterogeneity of each sort is arbitrarily limited to simplify the diagrammatic representation of the individual antibody molecules, so that there are shown only two valences, only two homologies, and for one of these homologies only two combining strengths.³ The combining sites A and A' are defined to have the same homology, *i.e.*, they are directed against the same antigenic determinant, but different combining strengths, so that one reacts more strongly than the other with the homologous determinant. The combining site B is defined to have a homology different from that of A and A' and a combining strength equal to that of A. Heterogeneity with respect to valence is illustrated by diagrams A, A', and B (each with one combining site) as compared with diagrams AA, A'A', BB, AA', AB, and A'B (each with two combining sites). Intramolecular heterogeneity with respect to homology is shown in diagrams AB and A'B; extramolecular heterogeneity with respect to homology is shown in diagrams A, AA, A', A'A', and AA' as compared with diagrams B and BB. Intramolecular heterogeneity with respect to combining strength is shown in diagrams AA' and A'B; extramolecular heterogeneity of this sort is illustrated by diagrams A, AA, B, BB and AB as compared with diagrams A' and A'A'.

It is hoped that the broad view of heterogeneity which we have taken in the above introduction will provide a suitable frame of reference for the experimental work which we shall now briefly consider.

The question of the valence of antibody has received considerable attention, since it has importance not only for theories of antibody formation but also for theories of the mechanism of antigen-antibody reactions. An interesting study which bears on this question is that of Pappenheimer (109), who showed that in the early period of immunization of a horse with ovalbumin the serum did not precipitate the antigen, but did inhibit the precipitation of the antigen with an antiovalbumin serum prepared in the rabbit. After a longer period of immunization a flocculating antibody appeared in the serum. The result was ascribed to the presence of

³ We are making these limitations and we are intentionally omitting heterogeneity with respect to kind of complementarity to avoid making the diagram unduly complex. It should be kept in mind that the antibody molecules shown in Fig. 4 are only a few of many possible kinds.

"univalent" antibody in the early antiserum. The same antibody was shown to add to a specific precipitate prepared with rabbit antiserum (60). While the result may be readily interpreted in terms of valence, it has also been suggested that the inhibiting antibody is a "weak" antibody and that it may not be argued, therefore, that precipitating antibody must be multivalent (11). It may be appropriate to mention here that Tyler (140) has apparently succeeded in converting precipitating antipneumococcal antibody to inhibiting antibody by photo-oxidation.

In experiments with antipneumococcal monolayers Porter and Pappenheimer (121) were able specifically to deposit alternate layers of antibody and polysaccharide on slides. This "sandwich" effect constitutes strong evidence that some antibodies are at least bivalent.

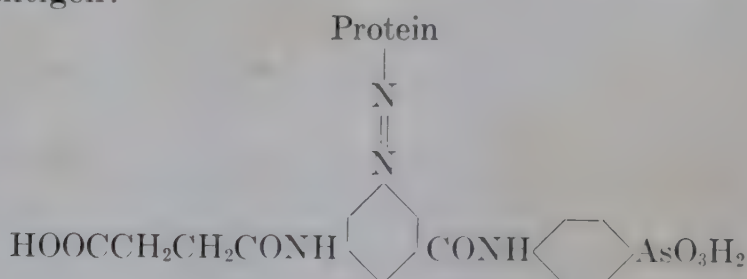
Pappenheimer, Lundgren, and Williams (110) carried out a study of the diphtheria antitoxin-toxin reaction in the ultracentrifuge and obtained evidence which led them to the conclusion that the antibody was bivalent.

The investigations of Pauling, Pressman, and Campbell (117) strongly suggest that antibody must be at least bivalent in order to build up a precipitating framework. They synthesized a simple antigen which combined one haptenic group R (arsanilic acid) and one haptenic group X (carboxylic acid); the antigen was therefore univalent with respect to each of these groups. Precipitation occurred when this antigen was added to a mixture of anti-R and anti-X, but not when it was added to either antiserum alone. The results, taken together with determinations of the mole ratio of antibody to antigen in the precipitate, were interpreted as support for the idea of bivalence of antibody and the framework theory of serological precipitation.

If antibody is bivalent, it may be supposed that the two combining sites of a given molecule can have different homologies. The first attempt to test this possibility experimentally was that of Haurowitz and Schwerin (53), who carried out a series of experiments with antiserum to arsanilic-azosheep serum. By absorbing the antiserum differentially with arsanilic acid antigens and with sheep serum globulin, they found antibodies for both the arsanilic acid group and the sheep serum protein, but the individual antibody molecules were specific for one or the other antigen and gave no evidence of containing combining sites for both types of antigen. From this they concluded that antibodies must be univalent, on the assumption that a multivalent antibody molecule would be

expected to contain combining sites of different homologies.

Previous evidence for the relative homogeneity of individual molecules was presented by Heidelberger and Kabat (55), who found that antibodies against SI, SII, and SIII (the type-specific polysaccharides of pneumococci of Types I, II, and III, respectively), isolated from an antiserum containing all three antibodies and purified by specific methods, showed no cross reactions in precipitation tests. This evidence is not so strong as that of Haurowitz and Schwerin, who used an antigen containing several determinants on the same molecule. The work of Landsteiner and van der Scheer (87) also indicated that the individual antibody molecules of an antiserum were directed against one or the other of two vicinal haptenic groups, the groups being succinilic acid and arsanilic acid, but not against the entire dihaptenic group. They used the antigen:



Their results also indicate that the antibody combining site has a relatively small size.

The importance for specificity of the area of the protein carrier to which an arsanilic acid group is attached was shown by Hooker and Boyd (64), who found that the reaction of antiserum with arsanilic acid was enhanced when the acid was coupled to tyrosine or histidine, which represent the groups of the protein carrier to which the hapten is coupled in the immunizing antigen. These results are in agreement with the results of other experiments (80) which show that, in general, an antiserum against a conjugated azoprotein antigen contains antibodies with combining sites directed against the hapten alone, the carrier alone, and the structural complex involving adjacent portions of the hapten and carrier simultaneously.

The question of the length of an artificial hapten which could be mirrored by an antibody was examined by Landsteiner and van der Scheer (88), who tested the cross reactions of pentapeptides made up of glycine and leucine in various combinations. They found, in substantiation of earlier work (85), that the terminal amino acid played a predominant role in precipitation reactions, while the

penultimate amino acid played a minor role, and the more proximal (to the carrier) acids were essentially ineffectual. However, inhibition tests gave some indication that the entire pentapeptide might be involved in the antigen-antibody reaction.

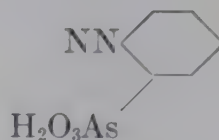
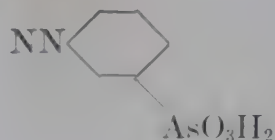
Although the area of the combining sites of antibodies may be relatively small it would seem likely that differences might be observed in antibody activity within this area if quantitative methods were sufficiently sensitive to detect the various degrees of fit for specific haptenic groups. With this in mind, Pauling and his group (118, 123, 116) have developed a theory which leads to the evaluation of two constants for each antigen-antibody-hapten system from a precipitation experiment in which an inhibiting hapten and a precipitating simple hapten are allowed to compete for antibody. One of these constants, K_0' , is an average effective hapten inhibition constant, representing the average bond strength of antibody and hapten relative to that of the antibody and antigen. The other constant, σ , is an index of the effective heterogeneity of the antiserum.⁴ The theory is based upon the assumption that the heterogeneity of the antiserum can be described as an error-function distribution in the free energy of the interaction of antibody and hapten in competition with antigen.

To constant amounts of precipitating antigen and antiserum (usually, although not necessarily, in the ratio giving maximum precipitation) there are added different amounts of hapten in constant volumes and the mixtures are allowed to come to equilibrium, a process which generally requires several days. A plot is made of the amount of precipitate, determined microanalytically and expressed as the fraction of that precipitate which forms in a reference mixture devoid of hapten, against the logarithm of the total moles of hapten added initially to the mixture. The quantity σ , the heterogeneity index, is evaluated graphically with the aid of a set of transparent templates, covering the range $\sigma=0$ to 5, at intervals of 0.5; values may be interpolated visually to 0.1 unit. This method of graphical analysis renders the evaluation of σ independent of the actual position of the experimental curve along the log total hapten axis. The theory leads to the evaluation of K_0' for a given hapten from the position of the experimental curve along the log total hapten axis in relation to the position of a similar curve prepared for a reference hapten, which is assigned a

⁴ σ , as used by Pauling and collaborators, is equal to $\sqrt{2}$ times the standard deviation, when the results are expressed in the form of the bell-shaped normal curve.

value of $K_0' = 1.00$. The antilogarithm of the displacement of the experimental curve along the log total hapten axis is equal to the normalized K_0' of the given hapten.

Values of σ and K_0' were obtained as described for over a hundred different inhibiting substances, using several simple precipitating antigens and rabbit antisera prepared against sheep or beef serum coupled with the following groups:



The results make it clear that σ is a function not only of the antiserum but also of the particular antigen and the particular hapten which are allowed to compete for antibody, so that σ expresses not a simple heterogeneity in the combining strength of different antibody combining sites with a given hapten, but a heterogeneity in the effectiveness of the competition of hapten with antigen for heterogeneous antibody combining sites. It is evident that a hapten which differs structurally from the haptenic groups of the precipitating antigen will not compete with equal effectiveness for all of the combining sites of a heterogeneous population, since it may compete poorly for combining sites which combine strongly with the antigen and more favorably for combining sites which combine weakly with the antigen; this follows since the latter combining sites may be more complementary to the hapten than to the antigen. The quantity σ is accordingly a reflection of the inequality of the competition of hapten and antigen for the several combining sites of a heterogeneous population. It would be desirable to have a method for determining the heterogeneity of an antiserum with respect to the combining strength of the several combining sites with a given antigen uncomplicated by competition, but this has not yet been provided.

5. The Stability of the Antibody Combining Site

The study of the effect of various physical and chemical treatments on the serological activity of antibody offers the promise of information regarding the types of forces which are responsible

for the maintenance of the structure of the combining site. If one finds that a given treatment leads to a reduction of antibody activity and if one knows from collateral experience the kinds of structures that are likely to be affected by the treatment, one may attempt to interpret the result in terms of the structure of the native (unaltered) combining site. This interpretation is difficult when the antibody activity is measured by one of the ordinary serological techniques such as precipitation, since these often depend on the assessment of phenomena which are not solely determined by the state of the combining site. Thus, if a bivalent antibody molecule were split into two univalent fragments without a structural alteration in the combining sites, the fragments might not be expected to precipitate with antigen, so that the ordinary precipitation test would not be a reliable index of the residual antibody activity.

Other features of the antibody molecule in addition to its content of active combining sites determine its precipitating properties. If a treatment were to affect any of these accessory features of the molecule, to interpret the result in terms of the activity of the combining sites would be misleading.

Considerable attention has been devoted to the study of the denaturation of antibody under a variety of conditions. In general, one may look upon protein denaturation as a change from relatively homogeneous specific structures to more random molecular arrangements. Of the many configurations which are accessible to the constituent polypeptide chains of a protein molecule, the native configuration is frequently not the most stable. The native configuration is maintained by primary chemical bonds, such as peptide bonds, which unite the amino acid residues in the polypeptide chains, and by secondary bonds, such as hydrogen bonds, salt bonds, and disulfide bonds, which fasten the chains to one another. The change from the native to denatured states, which must be attributed in part to the disruption of bonds such as these (98, 44), is manifested in the loss of biological activity, in the alteration of antigenic specificity, in the alteration of the viscosity, diffusion, and sedimentation properties, in the modification of the chemical reactivity of the molecule, and in other ways.

Since denaturation is frequently partially reversible and since antibody activity must be tested in the absence of denaturing agent, the state of the recovered antibody is not a reliable indicator of the state of the antibody in the presence of denaturing agent. The interpretation of denaturation experiments is further compli-

ated by the formation of insoluble material which is difficult to handle in tests of activity (cf. Erickson and Neurath (43)) and by the formation of soluble protein complexes (between antibody and antigen or between antibody and inert attendant protein) and of "inhibiting" antibodies. These complications render the usual tests of activity inadequate for the purpose of gaining information about changes in the structure of the combining site itself; and it became evident that much of the work will have to be repeated with purified antibody solutions with the aid of carefully controlled methods.

In the following discussion we have attempted to give some of the pertinent work dealing with the effect of heat, urea, guanidinium ion, enzymatic hydrolysis, surface spreading, irradiation, chemical substitution, and high pressures on antibody activity. For the fundamental aspects of the problems involved in protein denaturation, see reviews by Cohn, Edsall, *et al.* (28), Neurath *et al.* (104) and Anson (3).

Heat: The outstanding work on the effect of heat on antibody solutions has been carried out by Bawden and Kleczkowski (5) who found that the activity of rabbit precipitins and agglutinins was relatively unaffected by temperatures around 75°, that variations in the resulting apparent activity could usually be explained on the basis of the formation of protein complexes, and that the serological behavior of the complexed particles in precipitation tests depended on the type and quantity of proteins with which the antibody had complexed. Partially purified rabbit antibody preparations (euglobulins) when heated alone behaved very much like unheated antibody, and as more and more non-specific protein (serum albumin) was added to the system less and less specific precipitation was obtained, although the complexed antibody retained the ability to combine with antigen, as evidenced by its inhibition of the reaction between antigen and untreated antiserum. These authors also studied the apparent difference in heat stability of H- and O-agglutinins and concluded that there was no real difference in the stability of the two antibodies, but that complex formation between the respective antibodies and serum albumin interfered more with the reaction of O-antigen and its antibody than with the reaction of H-antigen and its antibody.

Essentially the same results were obtained by Jennings and Smith (69), who used horse antipneumococcus Type I serum. For example, they too found that the partially purified euglobulin fraction lost none of its specific precipitating activity when heated at 65° for 10 minutes under conditions which led to the

complete loss of this activity in whole serum. The addition of serum albumin, serum globulin, or casein to the euglobulin produced a loss in precipitating activity as long as there was no large excess of euglobulin. Although the heated antibody lost its precipitating power when mixed with non-specific proteins, its ability to combine specifically with antigen was shown by the inhibition reaction (*cf.* Lawden and Kleczkowski (8)).

Gerlough and White (50) studied the effect of heat on horse antitoxin and found that only about 50% of the neutralizing activity was lost after one day at 65° or seven days at 60°. Until further work is done on the significance of complex formation, experiments on the effect of heat on antibody activity will have to be interpreted with considerable caution.

Guanidinium ion and urea: Comparatively little work has been done on antibody denaturation with guanidinium ion and urea, although the effect of such compounds on protein structure may be relatively simple, involving only a disruption and formation of hydrogen bonds. From the work of Neurath, Cooper and Erickson (403) it may be assumed that treatment of globulin (presumably from horse serum) with urea and subsequent removal of the urea results in only slight changes in molecular configuration (depending upon the relative concentration of the various reagents) but that guanidinium ion produces a large amount of irreversibly denatured protein and a marked decrease in homogeneity (as based on solubility).

Proceeding from these initial observations these authors studied the effect of guanidinium ion on the antibody activity of globulin from serum of horses immunized against pneumococcus Type I, and found that after removal of the denaturing agent both the soluble and insoluble portions (the latter being dissolved in 2% NaCNS) gave specific precipitation reactions with SI polysaccharide (43). The antibody N/antigen ratios obtained at the equivalence point with the native antibody, "regenerated" antibody, native antibody in NaCNS, and "denatured" antibody in NaCNS were 4.6, 7.3, 1.7 and 1.0, respectively. Thus, the irreversibly denatured antibody still retained considerable activity and the regenerated form actually gave an increase in the amount of precipitable nitrogen. This latter finding has been confirmed by Campbell and Cushing (21) (see also Wright (152)) who found that under certain conditions of denaturation with 8 molar urea the amount of specific precipitate obtained with treated rabbit anti-albumin was greater than with untreated antibody preparations.

These results, which were similar to those of Bawden and Kleczkowski (8) dealing with heat denaturation, could be explained on the basis of the formation of complexes between antibody and non-specific protein. Solutions of purified antibody preparations showed no decrease in activity unless a relatively large amount of inert protein was added to the system, an amount sufficient to mask all of the combining sites on the antibody molecules. When small amounts of non-specific protein were added, the resulting complexes still contained a sufficient number of free combining sites to allow the formation of a precipitating framework. This enhancement of precipitation was also obtained when rabbit antipneumococcus serum was exposed to conditions of pH 4.0 or lower (145). The recent work by Wright (151) on the effect of urea on partially purified horse antitoxin indicated that some loss of neutralizing activity (Römer skin tests) was induced by urea in concentrations greater than 7 molar. A 40% reduction was obtained in 7.6 molar urea when the mixture was allowed to stand at room temperature for 48 hours.

In view of the difficulties introduced by complexing and by non-specific factors involved in *in vitro* reactions it seems likely that neutralization tests are superior to precipitation tests for the assay of the surviving antibody activity. In an earlier investigation, Pappenheimer, Lundgren and Williams (110) reported that urea had no effect upon the activity of horse diphtheria antitoxin, a result which could be explained on the basis of their use of too low a concentration of urea (3 molar). Wright stated that at least 5.7 molar was necessary to bring about a reduction in activity.

Enzymatic hydrolysis: The action of enzymes on antibody activity has been widely studied, first because of interest in the chemical nature of antibodies, and more recently because of the bearing of these studies on the purification of antibodies and on certain aspects of protein structure. In general, it has been found that the antibody activity is relatively stable to enzymatic action although the literature yields a confusion of results due to differences in methods employed in digestion and in testing for the resultant antibody activity.

By far the greatest amount of work has been done on the effect of proteolytic enzymes on diphtheria antitoxin, starting with the investigations of Pappenheimer and Robinson (111), Weil, *et al.* (146) and Pope (120). The latter investigators digested horse diphtheria antitoxin with an excess of pepsin at pH of about 4.0 for 4-24 hours at 37°, thereby reducing the heat-coagulable protein in the

mixture to about 25% of the initial amount, with a corresponding loss in antigenicity but no appreciable decrease in antibody activity.

Subsequent quantitative investigations by Pappenheimer, *et al.* (110) and Petermann and Pappenheimer (119) revealed that pepsin splits off an inactive portion of the antibody molecule in a plane perpendicular to the long axis, as evidenced by a reduction in molecular weight (from 184,000 to 113,000) and in axial ratio (from 7.0 to 5.3) and an increase in relative antitoxic activity depending on the amount of protein split off during the digestion. It is particularly interesting in relation to antibody structure that, in the case of antitoxin at least, the combining sites of a given molecule appear to be fairly well localized; this may account for the peculiar precipitation reaction between antitoxin and toxin.

Further studies by Northrop (106) on the tryptic digestion of diphtheria antitoxin led to the preparation of a highly active purified product which crystallized in poorly formed thin plates and had essentially the same physical properties as pepsin-treated antitoxin (126). Antitoxin is also stable in the presence of Takadiastase, as shown by the experiments of Coghill, *et al.* (27), who found that, although the antigenic activity of the antibody might be reduced, there was little effect upon the antibody activity. In contrast to the antitoxins, the antibodies to other antigens show considerable variation in resistance to proteolytic enzymes. For example, Rosenheim (125) reported that the H-agglutinins produced in the horse against *E. typhosus* showed a much greater resistance to the action of pepsin and trypsin than did the corresponding O-agglutinins; however, both types of antibodies were rapidly destroyed by activated papain. The studies of van der Scheer, *et al.* (143) with horse antisera against diphtheria toxin, tetanus toxin, and pneumococcus Types I and II confirmed earlier observations of the relative stability of the antitoxins and indicated the lack of resistance of the antipneumococcus antibody, which quickly lost its protective activity and to a lesser extent its specific precipitating power (see also Chow, *et al.* (25)).

Surface denaturation: Little study has been made of the surface denaturation of antibodies, although this phenomenon offers a basis for the most direct method for determining the effect of molecular unfolding on the activity of biologically active protein molecules. An excellent discussion of the fundamental aspects of protein monolayers has been given by Neurath and Bull (102). In considering studies on surface denaturation of proteins it is well to

note that extended films of most proteins, including antibodies, give film thicknesses of 8 to 12 Å. Consequently films of greater dimensions are not completely unfolded or contain appreciable amounts of adsorbed native protein. For this reason the first work on the surface denaturation of antibodies must be looked upon with interest only from the standpoint that alternate layers of more or less native antigen and antibody can be built up by the Blodgett technique.

Danielli, *et al.* (33) working with horse antipneumococcus Type II antibody found that antibody films adsorbed at air-water or oil-water interfaces gave no indication of specific combination with SII. The later work by Rothen and Landsteiner (128) gave rather conclusive evidence that well unfolded antibody molecules could combine with their corresponding antigens if suitable precautions were observed in detecting the reaction by film studies. In these studies purified rabbit anti-SI and anti-SII were spread at an air-water interface and then transferred in the usual manner to stearate-conditioned plates. The thickness of the serum globulin film was approximately 10 Å, which corresponds to a well-extended protein configuration. No appreciable increase in thickness could then be observed when the antibody film was exposed to a solution of antigen, but if the plate was then subsequently exposed to a solution of homologous antibody an increase of 40–60 Å was observed, indicating that antigen did combine with the initial antibody layer. The conflicting results of the earlier experiments of Danielli, *et al.* may be due in part to inherent differences between horse and rabbit antibody but more likely to methods involved in the detection of the antigen-antibody reaction.

Irradiation, chemical substitution and hydrostatic pressure: Proteins may be profoundly changed by irradiation, chemical substitution, and hydrostatic pressure, but the fundamental mechanisms involved are in general poorly understood. Irradiation with x-rays or ultraviolet light or oxidation with photosensitized dyes often results in the rapid disappearance of antibody activity. In this connection the recent work of Tyler (140) on the photo-oxidation of various antisera, in addition to confirming earlier reports, is of particular interest since he found that, although treated sera no longer gave typical antibody reactions such as precipitation, agglutination, protection or sensitization, they still had the power of specifically combining with antigen as evidenced by inhibition reactions. Thus, as has been shown for antisera denatured by other means, such as heat, the irradiated serum also tended to

inhibit or shift the zone of precipitation or agglutination between antigen and untreated antibody.

It was assumed that this inhibiting effect was indicative of the formation of "univalent" non-precipitating antibody; however, confirmation of this interpretation must await more quantitative data. Boyd (12) has also recently reported on the effect of photo-oxidation of human serum containing isohemagglutinins for group A cells and for Rh cells. Although the agglutinating activity of the serums was destroyed there was no evidence of the formation of inhibiting or "blocking" antibodies.

Studies on the effect of adding a variety of chemical groups to the antibody molecule have been made with the general result that antibody activity may be reduced or destroyed, depending upon the extent of the substitution. These reductions in antibody activity in many cases can be attributed to changes brought about by temperature, H-ion concentration, etc., and not to the actual introduction of substituents on the antibody protein. Groups such as azo, aldehydo, acetyl, iodo, and nitro and reagents such as ninhydrin, phenyl isocyanate, and fluorescein isocyanate have been studied, and although all tend to reduce antibody activity they have no unusual or specific effect on the antibody combining site. In fact, the antigenicity of the antibody molecule is usually affected to a greater degree than is the antibody activity.

Although considerable work has been done on the denaturation of protein with high pressures, relatively little attention has been given to the effects of this treatment on antibody activity. Recently Boyd (13) studied the effect of pressure on human anti-A, anti-B, anti-Rh, and strong "blocking" anti-Rh serums. The critical pressure for denaturation of anti-A, anti-B, and anti-Rh was 3,000 to 4,000 atmospheres and for the blocking anti-Rh antibody the critical pressure was about 5,000 atmospheres at which pressure the serum proteins begin to coagulate. It was apparent from this study that incomplete or blocking antibodies were not produced by such procedures and that the natural blocking antibodies were relatively resistant.

Sedimentation studies showed that pressure produced some aggregation of the serum proteins, since the final serum became quite heterogeneous with about 50% of the protein showing sedimentation constants of over 20 svedbergs. Much lower pressures (about 500 atmospheres) were found by Campbell and Johnson (22) to inhibit specific precipitation between rabbit antiserum and a simple precipitating dye antigen, but it would appear likely that this

result was due to an effect on the final aggregation and precipitation of the antigen-antibody complex rather than on the initial reaction. The same inhibiting effect was obtained for the denaturation of proteins with heat and alcohol (Johnson and Campbell (70)) and for the heat denaturation of *Staphylococcus* hemolysin (Johnson and Wright (71)).

6. The Conditions Affecting Antigenicity

The immunological tenet that antigenicity, the capacity of certain materials to induce antibody formation in animals, is explicable in terms of the chemical and physical properties of antigens leads naturally to the inquiry whether there are common to antigens any chemical and physical properties, or combinations of such properties, which distinguish them from non-antigens. One might suppose that it would be possible to prepare a list of such properties and that one could thereby discover antigens without recourse to animal experimentation.

Such a view would be mistaken, however, for it neglects that aspect of the total immunological process which makes antigenicity relative to the particular animal which is selected for injection. For example, the proteins, such as serum albumin, which occur in the circulation of an animal are not demonstrably antigenic in individuals of the same species,⁵ while it has been reported that proteins, such as lens proteins, which do not normally occur in the circulation are able to induce the production of antibodies in the same individual which supplied the antigen (auto-antibodies) or in other individuals of the same species (iso-antibodies). Other substances, such as the human blood-group antigens and the Rh antigen, are demonstrably antigenic in some individuals of a species but not in others; these individual differences have in general found an explanation based on inheritance. In the same category falls the difference in susceptibility of various human individuals to the several allergies. Furthermore, the variation in antibody response among different animal species is well known; for example, rats and guinea pigs, in contrast to rabbits and horses, are notoriously poor producers of precipitating antibodies; and, indeed, it is questionable whether invertebrates are able to produce antibodies, although there occur naturally in the blood of lobsters materials which simulate antibodies.

⁵ Some workers deny that antibodies are produced in this situation, while others maintain that antibodies are formed but are continually removed by the antigen which is constantly present; there is little direct experimental evidence supporting either position.

The recognition of species differences in the ability to produce antibodies as well as in the serological properties of the antibodies obtained finds application in the proper selection of the animal to be immunized when serological techniques are applied to the study of zoological relationships. Thus, tested with rabbit antisera, antigens supplied by closely related mammals may appear widely different, while antigens supplied by dissimilar species of birds may appear closely similar. As Landsteiner (80) points out, "this is, so to speak, a case of faulty perspective . . . (for) it may be understood why rabbit immune sera are very useful for revealing dissimilarities in the proteins of other rodents, while in the case of birds, if rabbit sera are employed, the lesser differences may be hidden by the predominant structural similarities of the bird proteins."

An additional feature of the concept of antigenicity is the technical arsenal of the immunologist in a given period, so that it is always possible for the application of new technical advances to bring into the category of antigens materials formerly thought to be non-antigens. Thus, the use of adjuvants (materials injected along with the antigen to enhance the antibody response) is becoming widespread (74). Furthermore, there has recently been reported (30) a new type of Rh antibody, one which is not demonstrable by the usual techniques of agglutination and inhibition (blocking), but which is detected by the agglutination which ensues when a rabbit anti-human globulin serum is added to a suspension of human red cells treated with the new Rh antibody. It seems likely that this "developing" technique for the detection of antibody will find wide application in other systems.

From the maze of experimental observations on immunizations there appears some suggestion of a few characteristics which seem to be fairly common to antigenic substances. Thus, the minimal molecular weight seems to be approximately 10,000 (clupein treated with phenyl isocyanate $\approx 5,000$, pneumococcus polysaccharides $\approx 10,000$, ribonuclease $\approx 15,000$). Smaller degradation products of proteins lack antigenicity, although Landsteiner (79) found that a hydrolytic product of silk fibroin (m.w. 600–1,000 by amino acid analysis) would combine with an antibody. Substances of smaller molecular weight have been found by Landsteiner and others (80) to be capable of inducing hypersensitivity but in most instances it is likely that these substances first combined with body constituents to form complex conjugated antigens.

From the standpoint of chemical composition one can only state

that most antigenic substances are proteins. There are, of course, many exceptions, and these are very interesting in that they may contain part of the secret of antigenicity.

There is much evidence that certain polysaccharides can stimulate antibody formation without being conjugated to proteins. For example, purified nitrogen-free polysaccharides (sensitivity of nitrogen analysis would limit maximum protein content to 0.015%) from parasitic helminths have been shown by Campbell (17, 18, 20), Melcher (95) and Oliver-Gonzales (107) to be fairly good antigens, capable of producing all of the typical antibody responses. The induction of an antibody response to certain pneumococcus polysaccharides has been recognized for some time (48, 155) and has recently been the subject of a quantitative study by Heidelberg, *et al.* (59) and by Walter, *et al.* (144).

The importance of aromatic groups to functional antigenicity has long been stressed but is doubtful. This idea has been developed because gelatin contains few or no such groups, and if it is sufficiently pure it does not cause the formation of antibodies. Furthermore, irradiation of proteins often results in a decrease in antigenicity (Henry (62)) as well as a liberation of tyrosine and histidine from the protein, which may also occur when proteins are subjected to mild alkali treatment and may be the responsible factor, rather than racemization, for reduction in antigenicity.

The direct approach to this problem by the introduction of aromatic groups into the gelatin molecule has been attempted many times with varying success. Antibodies formed against such conjugated gelatins either were extremely weak or were reactive only with other proteins containing the same haptenic group (63), in spite of the fact that R-azogelatin, for example, is a good precipitating antigen when tested against serum from rabbits immunized with R-azosheep serum. On the other hand, the ability of polysaccharides to induce antibody formation and the lack of this ability by proteins such as histones and certain animal nucleoproteins which contain aromatic amino acids indicate that such groups are neither necessary nor sufficient for antibody formation. In fact, there is a suggestive correlation of functional antigenicity with the carbohydrate content of proteins, although here again the studies of Clutton *et al.* (26) with gelatin coupled with the azide of O- β -glucoside-N-carbobenzyl oxytyrosine would throw some doubt on such a correlation since the resulting antisera gave very confusing weak reactions.

One other property which has often been mentioned as a char-

acteristic property of antigenic substances is digestibility. If true, the fact suggests several interesting factors in antibody formation. For example, it suggests that fragmentation is required of the antigenic molecule as a preliminary to antigenic action, and indicates that the normal body contains substances (enzymes) which have some general complementariness for the antigenic material. There is no doubt that all good protein antigens are digestible but the possible digestion of antigenic polysaccharides in the mammalian body must await further investigation. The excretion of such substances in the urine cannot be taken as an indication of their indigestibility, since many digestible substances, for example ovalbumin, rapidly pass through the glomerular filters and appear in the urine.

7. The Disparity Between the Moles of Antibody Produced and the Moles of Antigen Injected

Evidence that the molecular ratio of antibody produced to antigen injected is considerably in excess of unity has accrued chiefly through experiments designed to test the early hypothesis of Buchner, according to which specificity resulted from the incorporation of a portion of the antigen molecule in the antibody molecule. A review of these experiments has been given by Sevag (131). The principal difficulties in determining precisely the quantitative relation between antibody produced and antigen injected are: 1. only the demonstrably reactive antibody can be readily determined and the antibody in tissues other than the blood is neglected; 2. the formed antibody is subject to destruction by natural processes; 3. the actual amount of antigen which reaches the site of antibody formation is not known (*e.g.*, an unknown amount of antigen may be excreted; and 4. it is possible for subunits arising by degradation of the antigen to be effective in antibody formation.

Disregarding these unknown quantities, of which the first three tend to reduce and the last tends to increase the ratio which is being considered, the disproportionality between antibody produced and antigen injected is often considerable. For example, Pappenheimer (109) reported experiments with a horse which was immunized against diphtheria toxoid over a period of one month. At the end of this time, during which a total of 60 mg. of diphtheria toxoid had been injected, the concentration of circulating antitoxin in the serum was about 25 mg. per ml. An approximate calculation shows that the total circulating antitoxin exceeded 600 g., which is 10,000 times the weight of antigen injected. If 70,000 and

150,000 are accepted as molecular weights of the toxoid and antitoxin, respectively, the molecular ratio of circulating antibody to antigen injected is approximately 5,000.

On the other hand, the serum of a horse which was injected with a total of 1.9 g. ovalbumin over a period of nine months contained at the end of this time 0.37 mg. precipitable antibody nitrogen per ml., which corresponds to about 50 g. total circulating precipitable antibody protein. The weight ratio of circulating antibody to antigen injected is, therefore, about 25; and the corresponding molecular ratio is about seven, when 150,000 and 40,000 are taken as the approximate molecular weights of the antibody and antigen, respectively. If, 1,000,000 is taken instead as the molecular weight of the antibody, the molecular ratio reduces to 1. The molecular weight of the horse antiovalbumin was not determined directly but was assumed to correspond to that of diphtheria antitoxin on the basis of the resemblance between the ovalbumin-antiovalbumin and the toxin-antitoxin precipitation reactions.

8. The Relation of Antibody Production to Protein Metabolism

According to the generally held belief that the antibody molecule is a molecule of "modified" serum globulin, one may reasonably suppose that antibody formation is closely related to normal protein synthesis. For the immunochemist, an important question is the stage of synthesis of the antibody protein at which the serological specificity is introduced. It will be seen (below) that, while the recent theories of antibody formation agree in assigning the antigen a role of specifically modifying the course of synthesis of globulin, they differ regarding the stage of synthesis of globulin at which the antigen exerts its imputed effects.

For example, one theory is based on the supposition that the antigen specifically modifies the system of enzymes concerned with protein synthesis; another on the supposition that the antigen, in combination with a normal protoplasmic component, affects the order in which the amino acids are laid down in the growing polypeptide chain; and a third on the supposition that the antigen influences the manner of folding of a formed polypeptide chain. Direct experimental evidence in support of any of these suppositions is lacking at the present time, so that it is not possible to decide how much and in what way the normal course of globulin synthesis is modified in the formation of antibody protein.

The present knowledge of protein synthesis is too fragmentary to provide a basis for a detailed picture of the synthetic aspects of antibody production. Recently, however, significant experimental advances have been made in relating these two phenomena. Schoenheimer and his collaborators (129), using the stable isotope of nitrogen, N^{15} , have shown that, contrary to the classical belief, the proteins of the body are in a state of rapid flux, which is characterized by the simultaneous operation of processes of synthesis and degradation (*cf.* previous work by Borsook (9) and Whipple (92) and their collaborators). When an animal in nitrogen equilibrium is placed on a diet containing N^{15} , the isotope is quickly incorporated in the proteins of the body, the rate varying from one protein to another.

Under the conditions of the experiment, *i.e.*, the maintenance of nitrogen equilibrium, it is concluded that the rapid synthetic processes by which dietary N^{15} is incorporated in the proteins are balanced by equally rapid degradations. The participation of antibody protein in the metabolic reactions involving the N^{15} pool has been strikingly shown (130). When an actively immune animal was placed on a diet containing N^{15} , at a time when the concentration of circulating antibody was decreasing, the amount of isotope in the circulating antibody steadily increased, until the isotope was again omitted from the diet. It was concluded that the antibody continued to be formed during the period of antibody decline, and that the fall in concentration was an expression of the relative preponderance of the rate of destruction over the rate of formation. On the other hand, the isotope apparently failed to be incorporated in the antibody of a passively immune animal (61). This result does not unambiguously indicate that passively injected antibody fails to undergo changes involving nitrogen replacement, but may mean only that the structural changes which are involved in the incorporation of N^{15} in a passive antibody molecule are incompatible with the retention of its serological activity (see, for example, reference (124)).

Since antibodies are proteins, it may be supposed that the nutritional state of an animal has a large influence on its capacity to produce antibodies as well as on its resistance to those infections in which the antibody forms a significant part of the defense mechanism of the host. Cannon and his collaborators (24) have shown that severe protein deficiency, brought about by a prolonged low-protein diet, leads to a marked reduction in the ca-

capacity of animals for making antibody and that this capacity is rapidly restored when high-quality proteins, protein hydrolysates, or crystalline amino acids (147) (in a mixture modeled after the amino acid composition of casein) are provided in adequate amounts in the diet. It is interesting that the antibody-forming mechanism of rats is not irreversibly destroyed even after a long period (191 days) of feeding on a low-protein diet (148). These results have an obvious bearing on programs for the rehabilitation of starved populations.

The foregoing experiments, while pointing sharply to the intimate association of antibody formation with protein synthesis, provide little information regarding the exact nature of the relationship and leave unanswered the important questions of the manner in which antibody specificity is conferred on a globulin molecule and the nature of the deviations, if any, of antibody synthesis from the synthesis of normal globulin.

9. The Site of Antibody Formation

The identification of antibodies with the serum globulins and the prevailing conception that protein synthesis is a carefully controlled intracellular phenomenon have led to the supposition that antibody formation occurs in cells, and considerable attention has accordingly been devoted to the precise anatomical location of this process. It has long been known that when antigenic particles, such as bacteria, are injected into an animal the particles are rapidly engulfed and broken down by the phagocytic cells of the reticulo-endothelial system; and it has been commonly supposed that the same phagocytic cells are concerned with the synthesis of antibodies. While this supposition is not unreasonable, it has received only indirect support, and the precise role of the reticulo-endothelial system in antibody formation has not been elucidated.

Provocative results have recently been communicated which appear definitely to implicate the lymphocytes in antibody formation. Dougherty (36) and Harris (52) and their collaborators simultaneously demonstrated the presence in lymphocytes of antibodies in higher concentrations than in the corresponding immune serum or lymph. While this result may be interpreted to mean that the lymphocytes either produce antibodies or accumulate them from the surrounding lymph, the former alternative is preferred by Harris, *et al.* (52), who failed to observe any uptake of antibody by normal (non-immune) lymphocytes exposed to antibody-

containing fluids under various *in vitro* and *in vivo* conditions.

Moreover, it was shown (40) that following the injection of antigens into the hind feet of rabbits antibody appeared first in the lymph node nearest the site of injection and in the lymph leaving this node; in some cases the concentration of antibody in the leaving lymph was 100 times the concentration in the entering lymph. These results have led Ehrlich and Harris (41) to formulate a new hypothesis, according to which the lymphocytes are the site of antibody formation, and the reticulo-endothelial system is assigned the role of preparing certain antigens prior to their utilization by the lymphocytes.

Evidence that the lymphocytes may act as a storehouse of antibody protein was provided by Dougherty, *et al.* (37), who obtained large increases in circulating antibody by the administration of pituitary adrenocorticotropic hormone or adrenal cortical extracts to actively immunized mice or rabbits at a time when the titer of circulating antibody had fallen to zero. The appearance of released antibody in the circulation coincided with retrogressive changes in the lymphocytes, induced by adrenal cortical hormones.

10. The Persistence of Circulating Antibody After the Disappearance of Antigen

One of the most important but most confusing aspects of antibody production is the continued presence of antibody in the circulation after antigen can no longer be detected in the immunized animal. This observation raises the important question whether the antibody-forming mechanism continues to operate in the absence of antigen. Two factors complicate the analysis of this problem: first, it cannot be determined with certainty that the antigen is completely absent during the period of the continued presence of antibody in the circulation (the difficult proof of the negative); and second, it is not known whether the antibody continues to be formed or is merely released from surviving storehouses.

The long-lasting immunity which is a characteristic consequence of certain virus infections and the recovery of latent virus in some instances, long after disease symptoms had disappeared, support the conclusion that the level of circulating antibody in such immunities is based on the continued formation of new antibody in the presence of persistent antigen. This conclusion is further strengthened by the relatively short half-life (approximately two

weeks) of circulating antibody and the improbability of the survival of antibody storehouses over periods involving years.

On the other hand, the slow release of antibody from reservoirs may contribute to the maintenance of the level of circulating antibody over shorter periods of time. The possibility that the lymphocytes serve as a storehouse of antibody protein, which is released under the influence of adrenal cortical hormones and other agents (37), has been mentioned.

A related phenomenon is the accelerated response of the antibody-producing mechanism to secondary injections of a given antigen (specific anamnesis (23)). It is commonly known that the antibody response to a primary injection of certain antigens is characterized by a relatively long latent period, followed by the slow attainment of a relatively low concentration of circulating antibody, and then by a rapid decline in antibody concentration; in contrast, subsequent injections may be followed by a rapid response, the attainment of relatively high concentrations of antibody, and a slow decline (16). The accelerated secondary response forms the basis for the familiar "booster shots" which are employed in the maintenance of an adequate immune state against certain common human infections.

VI. THEORIES OF ANTIBODY FORMATION

It is remarkable that, in spite of the interest in immunity and immunological reactions, relatively few theories have been proposed for the mechanism of antibody formation. This is all the more remarkable when one considers that antibody formation is generally recognized as a fundamental biological phenomenon of which immunity is only one manifestation. For thirty years after the fundamental precepts of immunological reactions had been established only two noteworthy theories were proposed: namely, Ehrlich's theory of the existence of all antibodies as normal cell constituents, the overproduction of which was stimulated by the antigen; and Buchner's theory of the incorporation of antigen in the serum proteins. Ehrlich's theory was the first to be discarded, on the ground that normal tissues would not be expected to contain antibodies against such artificial and unbiological groups as phenylarsonic acid, etc. Buchner's theory was more recently proven untenable, when analytical methods became sufficiently sensitive to assure the absence of antigen components in the antibody molecule.

1. The Breinl-Haurowitz-Alexander-Mudd Theory of Antibody Formation

As a result of the increasing knowledge of the chemistry of proteins, and the growing conviction that antibodies are modified globulins, three similar theories of antibody formation were proposed almost simultaneously about 1930. These theories, which were concerned chiefly with the mode of acquisition of specific antibody configuration, were proposed by Breinl and Haurowitz (15), Alexander (1), and Mudd (100). They have such a striking similarity of content and approach that for practical purposes they may be considered a single theory.

This theory may be stated as follows. Normal globulin is formed from amino acids by an enzymatically controlled synthetic process which goes on in cells. We may consider that in the non-immunized animal the synthesis of globulin goes on in a normal intracellular environment and that the spatial relation of the constituent amino acids of the globulin molecule reflects the local idiosyncrasies of the normal environment. When antigen molecules are introduced parenterally into an animal they make their way to the site of globulin synthesis and introduce an abnormal feature into the atmosphere in which synthesis is occurring. In the words of Mudd:

“As this synthesis (of globulin) proceeds in the presence of antigen, we may reasonably suppose some of the simpler structural units (*e.g.*, peptides) will be combined, either stoichiometrically or by absorption, with the antigen. Further synthesis, for instance by coupling of amino acids to the peptide, we suppose will now occur in an orienting environment, namely the antigen-protoplasm interface. Any ‘building-stone’ it is assumed can be added to the growing antibody molecule only if it conforms to the spatial and chemical requirements of the interface in which synthesis is proceeding. In brief the chemical groupings coupled to the molecule undergoing synthesis at the antigen surface must be adapted spatially and in their chemical affinities to the antigen surface at the region in which coupling occurs. The completed antibody molecule will therefore possess to some degree a stereochemical correspondence with the antigen for the reason that each structural unit has been selected and oriented, at the moment of synthesis into the antibody molecule, to fit the local configuration and affinities of the antigen surface.”

The important feature to note about this theory is that the antigen is assumed to affect the order in which amino acids and peptides are laid down in the growing globulin molecule. Beyond further suppositions that the antigen is made available to direct the synthesis

of more antibody by dissociation of the antigen-antibody complex (Mudd) and that the duration of immunity is related to the persistence of the "antigen-catalyst complex" (Alexander), the Breinl-Haurowitz-Alexander-Mudd theory has little to say. Indeed, the knowledge of the accessory features of antibody production was so fragmentary in 1930 that one could not reasonably expect a comprehensive detailed theory to have issued at that time.

The situation was different in 1940. During the intervening decade the application of powerful physico-chemical tools to the study of protein structure and of quantitative absolute methods to the study of serological processes led to significant advances in our understanding of the over-all structure of antibodies and the mechanism of antigen-antibody reactions. The stage was set for new theories, and two were forthcoming, one by Pauling (112) and one by Burnet (16).

2. Pauling's Theory of Antibody Formation

Pauling presented a detailed "theory of the structure and process of formation of antibodies" which departed from the earlier theories in the assumption that "all antibody molecules contain the same polypeptide chains as normal globulin, and differ from normal globulin only in the configuration of the chain; that is, in the way that the chain is coiled in the molecule." He was led to this reasonable assumption (*cf.* Rothen and Landsteiner (127)) through the difficulty of formulating a detailed reasonable mechanism of the process of antibody formation based on the antigen-induced alteration of the order of amino acid residues in the polypeptide chain. In view of the large number of configurations which are accessible to a single polypeptide chain, the new assumption provided an adequate basis for antibody specificity. Invoking the rule of parsimony Pauling assumed that an antibody molecule has at most two active regions and he presented a scheme whereby an extended polypeptide chain, of which the two ends are able to assume any of a large number of configurations and the central portion only one (characteristic of the normal globulin), becomes an antibody molecule under the influence of an antigen molecule acting as a template. The essential features of this process are shown diagrammatically in Fig. 5. By deriving the consequences of this theory, Pauling was able to account for numerous immunological observations, among which a few, pertinent to our discussion, may be mentioned.

The theory, expressly designed to account for the formation of a bivalent antibody molecule under the influence of a single antigen

molecule, allows for the formation of univalent antibody molecules since the manner of folding of one of the two ends of the polypeptide chain may under some conditions be relatively unaffected by the antigen. Moreover, since the surface of an antigen molecule may be considered to present a mosaic of determinant structures, and since

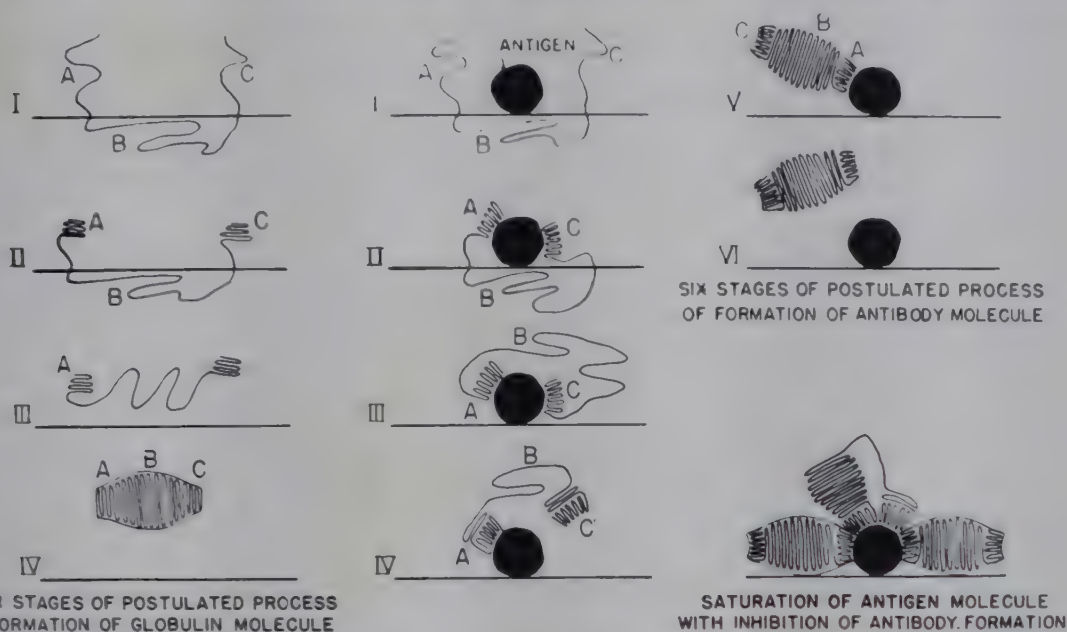


FIG. 5. Diagrams representing four stages in the process of formation of a molecule of normal serum globulin (left side of figure) and six stages in the process of formation of an antibody molecule as a result of the interaction of the globulin polypeptide chain with an antigen molecule. There is also shown (lower right) an antigen molecule surrounded by attached antibody molecules or parts of molecules and thus inhibited from further antibody formation. (Reproduced by the author's permission.)

the polypeptide chain end may fold to a varying extent before it comes under the influence of the antigen, there is provided a basis for intramolecular and extramolecular heterogeneity of antibody with respect to homology, combining strength, and kind of complementariness (see above). The progressive changes in the character of the antibody (intensity of cross-reactions, broadening of the equivalence zone, etc.) which occur during the course of immunization are not considered in detail.

The similarity in the physico-chemical properties of antibody and normal globulin has an evident explanation in their derivation from a common precursor. The postulated invariant configuration of the central proportion of the molecule (antibody or normal globulin) accounts for the antigenic similarity of antibody and normal globulin. The apparent non-antigenicity of the antibody combining site is explained on the basis that the structurally heterogeneous combining sites of an antibody population induce the formation of anti-

antibody molecules each of which combines effectively with one of the (antigenic) combining sites but not with another, so that in a precipitation reaction between antibody and anti-antibody the combining sites of the antibody (acting as an antigen) are not significantly effective in promoting the formation of a framework precipitate.

In answer to the question of what makes a substance an antigen, Pauling submits the following criteria for antigenic activity:

"1. The antigen molecule must contain active groups, capable of sufficiently strong interaction with the globulin chain to influence its configuration.

"2. The configuration of the antigen molecule must be well-defined over surface regions large enough to give rise to an integrated antibody-antigen force sufficient to hold the molecules together.

"3. The antigen molecule must be large enough to have two or more such surface regions, and in case that the antigenic activity depends on a particular group the molecule must contain at least two of these groups. (This criterion applies to antibodies effective in the precipitin and agglutinin reactions and in anaphylaxis.)"

When a bond of average strength forms between antigen and antibody during the process of formation of antibody, the complex will in time dissociate and the antigen will be free to affect the folding of another polypeptide chain. If, however, the antigen contains numerous strongly reacting groups, the antigen-antibody bond, arising during the formation of antibody, may persist for long periods of time and only a low concentration of antibody will be built up in the serum. This consideration leads to the conclusion that;

"An antigen containing weak groups will in general be a good antigen, whereas one containing many strong groups will be a poor antigen, with respect to antibody formation."

Continuing the argument,

"To achieve a sufficiently strong antibody-antigen bond with an antigen containing only weak groups a large surface region of the antigen must come into play, whereas with an antigen containing strong groups only a small region (in the limit one group) is needed. Hence antibodies to antigens containing strong groups show low specificity (*i.e.*, extensive cross-reactions), and those to antigens containing weak groups show high specificity."

To account for the disproportionate production of antibody and for specific and non-specific anamnesis, Pauling assumes that:

"Following the synthesis of an antibody a mechanism comes into operation in the cell to facilitate the removal of the antibody from the

antigen, perhaps by changing the hydrogen-ion or salt concentration or dielectric constant."

Such a mechanism would conceivably be independent of any serological relationship between antigens, so that a heterologous antigen would lead to the dissociation of persistent complexes formed between antibody and a previously injected antigen. The precise mechanism by which this effect is achieved is left poorly defined.

It is natural that a detailed theory such as this should have been subjected to criticism. The relative stability of antibody activity with respect to various denaturing influences (see above) has led some workers to question the tenability of Pauling's primary hypothesis according to which antibody specificity is based on a particular folding of a polypeptide chain,⁶ rather than on an altered amino acid composition or sequence. For example, Erickson and Neurath (42) reported the recovery of active antibody following denaturation in 8 *M* guanidine-HCl, in the absence of antigen, and suggested that "contrary to Pauling's hypothesis the difference between antibody globulin and normal globulin is not merely one of steric arrangement but probably one of amino acid composition."⁷ In a recent communication (43), however, they allowed the possibility that "the serological activity (of antibody) is associated with structural regions which are unaffected by the denaturing process." Moreover, they suggested that additional combining sites may be buried within the native antibody molecule and that these may be liberated during denaturation.

The observation by Rothen and Landsteiner (128) that antibody films retain serological activity has been interpreted by Neurath, *et al.* (104) (see, however, reference (43)) as evidence of "the independence of antibody activity to a specific structure," in spite of the proffered explanation of Rothen and Landsteiner that antibody activity may reside in an "arrangement due to 'secondary' linkages, in a plane" and that this arrangement is resistant to the disruptive effects of spreading at surfaces.

Perhaps the most serious criticism is that of Burnet (16), who has pointed out that Pauling's handling of the processes auxiliary to antibody formation, such as the attendant increase in normal globulin during immunization and the anamnestic phenomena, is

⁶ Support for this hypothesis has been provided by the manufacture of antibodies *in vitro* by a method involving the denaturation of gamma globulin and its renaturation in the presence of a suitable template (114) (*cf.* 49). The artificial antibodies differ from immune antibodies in having a relatively high valence.

⁷ Objections to this conclusion were offered by Wright and Pauling (153) and Campbell and Cushing (21).

rather weak. Our own feeling is that Pauling was concerned not so much with the elucidation of these auxiliary processes as with the clarification of the essential features of antibody structure and antibody formation; and while his theory may by no means be regarded as proved, its simplicity, directness, and clarity have made it the most stimulating conception of antibody formation so far proposed.

3. Burnet's Theory of Antibody Formation

Burnet himself provided the second recent theory of antibody formation (16). Chiefly concerned with the difference in response to primary and secondary injections of antigen (specific anamnesis), the progressive changes in antibody (decrease in specificity, etc.) during the course of immunization, and the occurrence of long-lasting immunity in the apparent absence of antigen, he proposed the idea that the role of the antigen is to modify the system of proteinases which are concerned with globulin synthesis. He assumes that the proteinases are more or less permanently modified by the antigen, the modified proteinases having a structure complementary to that of the antigen and in this respect being primary "antibodies," and that the modified proteinases are able to replicate themselves, the replicas retaining the complementary configuration. These replicas (or parts of them) are liberated into the body fluids and are demonstrable as antibodies. In his words:

"... the primary modification of the proteinase within the antibody-producing cell may be pictured as such a re-orientation of its atomic structure as is necessary to allow its effective hydrolytic action on the foreign antigen. The antigen is disintegrated into substances which can either be utilized for cellular purposes or excreted. The modified proteinase remains, and retains its modification during subsequent production of complete or partial replicas. It is not, however, in the nature of living substance to remain unaltered after modification. In the absence of further contact with antigen successive new formation of replicas of the modified proteinase will probably gradually eliminate the impression, and ultimately normal globulin will be produced. In the intermediate stages, low-grade antibody which unites with the antigen less readily will be produced. . . . Further contact with the antigen will, on the other hand, intensify the impression. If a new antigen particle or molecule enters a cell containing one or more proteinase units already modified by previous contact with the same antigen, union will take place more readily, and an opportunity will be presented for modification of other aspects of the proteinase to occur. There is also the possibility that on account of the more rapid hydrolytic action of the modified proteinase partially disintegrated antigen may be in a position in its turn to

modify the proteinase. Determinant groups not effective in the primary antigenic stimulus could thus come into play. In other words, successive contacts with antigens have an additive effect in modifying the proteinase units."

While it is denied that a new proteinase unit is synthesized in spatial contact with the antigen, the precise mechanism by which the antigen exerts its effect is not clarified.

This theory is inherently plausible, especially in view of the analogy between the modified proteinase, or reproducing "antibody," and the bacterial adaptive enzymes, but the experimental support is rather weak. The chief argument which Burnet adduces is based on the difference in response to primary and secondary injections of antigen, and especially the quantitative course of the secondary response. When the log of the antibody titer produced in response to a second injection of antigen was plotted as a function of time, he obtained a curve one portion of which could be fitted with a straight line. Burnet concluded that this linear log titer-time relationship indicates autocatalytic proliferation of the antibody-producing mechanism. It is our feeling that the same data are fitted equally well with a sigmoidal curve of continuously changing slope, and that the form of the curve may not be adduced as evidence of the involvement of an autocatalytic mechanism. Moreover, even if the linear log titer-time relationship is granted, there is ample opportunity for variability in the complex over-all phenomenon of antibody formation, and it is possible that Burnet's data show no more than that such a variability exists.

We feel, however, that Burnet has made a valuable attempt to interpret highly complex biological phenomena and that his theory is somewhat complementary to Pauling's theory in this respect. Where Pauling's significant contribution is his provision of a structural conception of the formed antibody molecule, consistent with its serological and chemical behavior, and a clear hypothetical mechanism for the formation of such a molecule in spatial contact with the antigen, Burnet has emphasized important biological aspects of antibody production and has neglected the physical properties of the antibody molecule. The two theories disagree chiefly in the roles which they assign to antigen in the process of antibody formation. If the possibility is allowed that the antigen induces by direct contact, by a mechanism such as described by Pauling, the formation of a proteinase endowed with autocatalytic properties, a manner of combining the two theories is provided.

VII. CONCLUSION

In the foregoing sections the authors have attempted to present some of the pertinent facts and current speculations regarding antibodies which may be of particular interest to protein chemists. Many important problems still await solution by the combined efforts of the chemist and the biologist, and it is appropriate in closing to consider briefly some of the problems which are suggested by the preceding discussion.

One of the most important problems at present is the heterogeneity of antibodies. In the past few years it has become obvious that a single molecular antigen may stimulate the formation of a variety of antibody molecules, differing from one another in the number of serologically active centers (valence), in the precise portion of the antigen toward which the combining sites of the antibody are directed (homology), and in the strength of combination and kind of complementariness of the combining sites. There is a need, therefore, for methods for the detection, estimation, and study of antibodies (*e.g.*, non-precipitating antibodies) which are not readily perceived by the conventional immunological techniques. While current quantitative methods are suitable for determining the amount of antibody which precipitates with antigen under given conditions, by no means may it be assumed that the precipitating antibody represents all of the antibody of an antiserum. In extreme instances, *e.g.*, Rh "blocking" serums or certain reaginic serums, the antibody may be completely non-precipitating, so that specialized techniques are required for its demonstration. It seems certain that ordinary antisera, of the precipitating sort, contain non-precipitating antibodies in addition to the precipitating antibodies and that the former must have an effect on the estimation of the latter. It is apparent, therefore, that the notion of heterogeneity must be given an important part in the further development of our conceptions of antibodies, their formation, and their reactions.

Studies of the various features of antibodies will be considerably aided by the use of purified antibody solutions, prepared in high yield and purity by methods such as have been described above. These solutions will be especially useful in investigations of the amino acid composition of antibody molecules and in studies of the chemical and physical properties of the combining sites. Results of such investigations will have an important bearing on the theory of antibody formation; and, until more is known about the detailed nature of the combining site, about the mechanism of synthesis of

antibody globulin, and about the level of synthesis at which antibody activity is acquired, important parts of the current theories must be regarded as pure speculation.

The application of isotopic tracers to the study of immunological problems should be extended in attempts to determine the origin and fate of antibodies and their role in immune reactions. Little is known about the functional or biological significance of antibodies and their contribution either to the defense against disease agents or to the conspiracy to produce disease, such as in the development of hypersensitivity. Only rarely is the precise role of antibody clear; *e.g.*, in the immune response to parasitic helminths, the antibody-antigen reaction is obviously responsible for the death of the parasite (Taliaferro (132a)). In other instances antibodies bring about subtle changes in the "integrity" of the surface membranes of cellular antigens, as evidenced by lysis or alteration in the susceptibility of the cells to drugs, but the underlying mechanisms are not understood.

Antibodies have many characteristics in common with the bacterial adaptive enzymes. These similarities may be superficial and spurious, but they suggest the fundamental ability of organisms to adapt themselves quickly to changing environments; and while antibodies have no apparent enzymatic activity, it is conceivable that they represent a part of an adaptive enzyme system or a vestige of such a system, handed down from evolutionary biochemical ancestors.

ADDENDUM

For certain developments subsequent to the preparation of this chapter the reader is referred to the following references:

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Chapter XII

BIOCHEMICAL APPLICATIONS OF PROTEINS AND PEPTIDES

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I. INTRODUCTION

IT IS THE PURPOSE of this chapter to illustrate the use of proteins and protein products above the level of complexity of the amino acids for conferring immunity, correcting endocrinological disorders, combating a variety of diseases, and for certain special ex-

perimental purposes in biology. The list of applications is not intended to be exhaustive and only the major examples will be discussed for reasons of economy of space and of interest. The proteins that are used biochemically include the plasma proteins, hormones certain antibiotics, and the viruses. Enzymes, which offer many examples of biochemical application, will not be considered here because they are too numerous to be considered adequately without unduly lengthening this volume. It is felt that they should be discussed in a separate treatise.

1. Functions of Proteins

An insight into the kinds of applications to which proteins can be put can be derived from a consideration of the nature of the functions performed by this class of substances. A survey of the functions of proteins is given below:

a. As structural elements

In animals, particularly, proteins form the chief structural elements of the body. The mineral matter of the bone is held together by a collagenous protein. The individual bones are joined by proteinaceous connective tissue, and the elasticity of the blood vessels is derived from the protein elastin. The internal organs (heart, liver, kidney, intestine, etc.) and the musculature of the body are composed chiefly of protein, the main protein constituents of muscle being myosin, globulin X, and myogen (4). The protective skin, hair and nails are formed from the protective protein, keratin.

b. In biocatalysis

The proteins are of outstanding importance in the control of the chemical reactions of organs through the agency of enzymes and hormones. The almost innumerable stepwise reactions that are essential for vital function are apparently all initiated through enzyme action. Some enzymes, such as the proteinases, amidases, carbohydrases and esterases appear to be composed only of amino acid residues. The aerobic oxidases, the dehydrogenases, and the phosphorylating enzymes may contain prosthetic groups that are not amino acids and that determine the general nature of their activity. The protein moiety, however, determines the specificity of action.

In plants, the property of photosynthesis is intimately related to specific proteins, and it appears probable that the photosynthetic pigment, chlorophyll, functions as the prosthetic group of a chlo-

rophyl-protein catalyst. A comparable situation is found in higher animals, where the iron-porphyrin containing hemoglobin is essential in carrying oxygen for the respiration of the organism.

In animals, such fundamental processes, as growth, sexual activity, rate of energy metabolism, carbohydrate metabolism, blood pressure, kidney excretion, and others, are regulated wholly or in part by hormones. A considerable number of these hormones have been shown to be proteins or polypeptides. Examples are insulin of the pancreas, concerned with carbohydrate metabolism; thyroglobulin of the thyroid, concerned with energy metabolism; the blood pressure raising hormone of the posterior pituitary, pitressin; and the different hormones derived from the anterior pituitary gland: growth, lactogenic, gonadotropic, thyrotropic, and adrenocorticotropic.

c. In osmotic and neutrality regulation

The blood volume and extra-cellular water balance of higher animals are dependent upon the osmotic effect of the plasma proteins, serum albumin in particular. Similarly, the state of neutrality and constancy of pH required by the animal organism is mainly supplied by the buffering capacity of the hemoglobin of the erythrocytes.

d. In immunity

Proteins are highly important for the immunity reactions of the animal organism. Proteins are particularly suited to exhibit antigenic activity. Antibodies that circulate in the blood stream are found to be associated with the globulin fraction of the plasma proteins, particularly the γ -globulin. All present evidence indicates that these antibodies are modified plasma globulins.

e. As disease carriers

Individual proteins are of great importance in many disease processes. Outstanding are the viruses, which are the incitors of many important diseases of plants and animals. Information that viruses are individual proteins came from the study of diseased tobacco leaves, induced by the tobacco mosaic virus. This virus was crystallized and shown to be a high molecular weight nucleoprotein (109). Isolations of other plant and animal viruses soon followed.

Another manner in which proteins are concerned in infectious diseases is in the toxins produced by many pathogenic microor-

ganisms. Examples are tetanus and diphtheria. Several of these toxins have been isolated in a pure state and have been shown to be protein in nature.

Also, it is extremely interesting that certain of the antibiotics that counteract the spread of disease are peptides.

f. In heredity

Proteins play important functions as determinants of heredity. All present evidence points to the chromosomes and genes of the sex cells being nucleoproteins.

g. In nutrition

Proteins are the source of the indispensable and dispensable amino acids for the nutrition of animals. In severe malnutrition and in conditions where there is inability to ingest food, the protein nutrition of man can be maintained by administration of homologous blood plasma or by injection of protein hydrolysates.

II. PLASMA PROTEINS

1. Functions of Plasma Proteins

The blood plasma serves many important functions in the physiological economy of the animal body that are dependent on the special physicochemical and biological characteristics of the different proteins of the plasma.

The better known of these functions are enumerated below:

a. *The maintenance of blood volume* and the fluid balance of the body is dependent upon the osmotic activity of the serum proteins, chiefly the albumin.

b. *The coagulation of the blood* affords protection against loss of blood from wounds or bleeding due to other causes. The better known protein fractions of blood plasma that are concerned with the coagulation reaction are prothrombin, which is elaborated in the liver and which is converted to thrombin through the activating action of thromboplastin and calcium ions; fibrinogen, which is transformed to the insoluble protein of the clot, fibrin, through a catalytic reaction with thrombin. In addition, a globulin component (Ac-globulin) has recently been demonstrated which accelerates the formation of thrombin, and another one which causes lysis of fibrin, commonly named plasmin. The blood-clotting mechanisms have been summarized by Ware and Seegers (117) by the following equations:

- (1) Prothrombin + Thromboplastin $\xrightarrow{\text{Ca}^{++}}$ Thrombin
- (2) Plasma Ac-globulin $\xrightarrow{\text{Thrombin}}$ Serum Ac-globulin
- (3) Prothrombin + Thromboplastin $\xrightarrow[\text{Serum Ac-globulin}]{\text{Ca}^{++}}$ Thrombin
- (4) Fibrinogen $\xrightarrow{\text{Thrombin}}$ Fibrin

c. *The circulating antibodies of the blood plasma* are valuable for protection against many diseases. All present evidence indicates that the antibodies of the blood are modified plasma globulins.

d. *The specific binding properties of the plasma proteins* are useful for the interchange and transport of many important compounds in the blood. Important lipids such as cholesterol, bile pigments, phospholipids, fatty acids, and the steroid hormones are found present in plasma in close association with the globulins, particularly two specific proteins, one in the α - and the other in the β -globulin fractions. β -Globulin also contains a pseudoglobulin component, named siderophilin, which is specifically responsible for the iron-binding capacity of blood serum (7, 37, 56, 67, 100).

Serum albumin has a marked ability to combine reversibly with a large variety of water soluble compounds. These include the anions and cations of many acid and basic dyes, the anions of aliphatic and aromatic fatty acids, alkyl sulfonic acids, sulfonamides, acetylated amino acids, naphthaquinones, and many others.

e. *Plasma proteins* serve a nutritive role as a reserve store of protein. In severe protein deficiency and in subjects who cannot ingest food, injection of plasma is beneficial in combating the effects of hypoproteinemia.

2. Fractionation of Plasma Proteins

Fractionation of blood plasma primarily offers the important clinical advantages of separating the individual plasma protein components and making each one available for its most advantageous specific use. As summarized by Edsall (30), the advantages of fractionation are:

(a) For a specific clinical condition usually a specific protein component of the plasma is essential, not the whole plasma. Thus the γ -globulin fraction which contains many antibodies is of value for passive immunization against certain infections or in their treatment, but γ -globulin is relatively ineffective in combating

shock, owing to its low osmotic activity. Serum albumin, on the other hand, is highly effective in the treatment of shock, but has no value for immunization. A specific plasma fraction has a far higher activity with respect to its specific function than the whole plasma from which it was derived. Consequently, the size of the dose is greatly decreased, and the therapeutic effectiveness greatly enhanced. This may be decisive for making possible the use of a plasma fraction for a clinical purpose for which whole plasma would be totally ineffective.

(b) Fractionation of the plasma proteins is important for reasons of stability and preservation. Whole plasma contains proteolytic enzymes which slowly digest the rest of the plasma proteins. The separated and purified fractions of albumin and γ -globulin are free of these enzymes and thus remain stable in aqueous solution for periods of several years. The fractions which contain the proteinases can be preserved dry by dehydration from the frozen state. The conditions for the preservation of the different plasma protein components in a state of maximum stability with a minimum of denaturation differs greatly. Serum albumin is strongly stabilized against heat denaturation by the addition of dilute sodium caprylate or the sodium salt of acetyl tryptophane.

γ -Globulin, however, is not stabilized at all by these compounds, but it is stabilized against denaturation by glycine and by certain simple sugars.

(c) Most of the separated plasma protein fractions can be redissolved after being dried from the frozen state to give clear active solutions. Whole plasma, however, contains lipoprotein in the β -globulin fraction which, upon drying, tends to disintegrate, so that, upon again being dissolved, the fatty material is released and the protein material becomes partly insoluble. By fractionation, the lipoproteins are prepared without drying and give clear aqueous solutions.

(d) By fractionation some of the plasma proteins may be converted to special products for new specific uses. Thus, fibrinogen may be converted to fibrin foam for use in hemostasis, fibrin film for use as a dural substitute, and fibrinogen plastics. The prothrombin may be converted to thrombin for use as a hemostatic agent.

Administration of human blood plasma and the separated plasma protein fractions have well-defined medical and public health purposes that will now be considered. Critical deficiencies of the plasma proteins as a whole or of specific components often develop as a result of disease processes or of accidents. It is medically important

to correct the lack of the plasma proteins, and more and more this is being done by the intravenous injection of plasma or its specific protein components.

3. Regulation of Blood and Body Fluid Volume

The maintenance of blood volume is due chiefly to the balance between the hydrostatic pressure of the blood in the capillaries (produced by the contraction of the heart) which tends to expel fluid from the blood, and the osmotic pressure of the plasma proteins which tends to draw fluid from the tissue spaces into the blood vessels.

The osmotic pressure of the plasma proteins results from the relative impermeability of the capillary membranes to colloids. It functions between the blood vascular system and the interstitial spaces. The crystalloidal constituents, particularly ions, play little part in this function because of the high degree of permeability of the capillaries to them, whereas the crystalloidal ions, because of selective permeability, are of the utmost importance for the regulation of the balance between extracellular and intracellular fluids.

Serum albumin is present in twice as high a concentration as globulin in blood; it has about half the molecular weight and carries a higher negative charge. Consequently, it is of major importance in maintaining the colloidal osmotic pressure.

In conditions where there is an acute loss of total plasma protein, transfusion with reconstituted dried plasma has been extensively employed and is of great value. Such transfusions have been used with great success in severe burns, intestinal obstruction, peritonitis, pneumonia, and in shock from other causes (32, 33). In conditions where there has been a loss of erythrocytes, it is desirable to administer transfusions of whole blood so that both the red corpuscles and the plasma proteins are replenished (33).

Concentrated human serum protein is highly effective in conditions involving shock for reasons already mentioned and was developed to meet the need of specialized combat units, such as landing parties, paratroops, seabees, and commandos (23, 61, 118). The Standard Army and Navy Package of Serum Albumin consists of 100 ml. of solution containing 25 gm. of human serum albumin in 0.3 *M* sodium chloride at a pH of approximately 6.8, preserved with 1:10,000 dilution of merthiolate. The albumin in this preparation is adequately thermally stable. For special civilian use, it is often desirable to prepare the albumin in much lower salt concentration or in a salt free medium such as glucose.

Human serum albumin has been tested in civilian medical practice, but, because of high costs, its administration is severely restricted. Among the advantages of serum albumin is that although it comprises only about 60% of the protein of plasma, it accounts for approximately 80% of the colloid osmotic pressure. Serum albumin is extremely stable and very soluble in water or in solutions of various crystalloids. The relatively greater symmetry of the albumin molecule than that of the globulins imparts a low viscosity to concentrated albumin solutions, which is more favorable to maintenance of the circulation of the blood (30).

Measurement of osmotic pressure indicated that one gram of albumin holds about 18 ml. of water in the blood. Consequently, 25 gm. of albumin represents the osmotic equivalent of around 500 ml. of citrated plasma (23, 61, 118).

The immediate response of patients to injection of concentrated albumin is a rapid fall in hemoglobin concentration and hematocrit and a subsequent increase in serum protein concentration, indicating the transfer of extravascular fluid into the circulation. Where the patient is markedly dehydrated the results obtained are unsatisfactory unless saline solutions also are administered.

Highly satisfactory results have been obtained with human serum albumin in the treatment of traumatic, hemorrhagic, and operative shock. In shock associated with infection, the results have been unsatisfactory, and in patients with liver cirrhosis, the improvement was only temporary (32, 33).

In conditions of chronic hypoproteinemia, very large amounts of albumin are required to raise the level of the serum albumin. Similarly, in nephrosis good results are obtained with large doses. In patients with edema, plasma or albumin therapy may be of value for a period to induce diuresis. It may be concluded that administration of human serum albumin is chiefly indicated to tide a patient over an emergency and maximal dosage in a minimum period of time should be administered (32).

4. Immune Sera

The use of convalescent serum for prophylaxis against a variety of infectious disease is a common medical practice. This treatment is based upon the increase in the concentration of antibodies of the blood plasma resulting from successful recovery from these diseases. It is now fairly certain that the antibodies are associated with or actually consist of globulins. Large quantities of globulins were made available in the fractionation of normal human plasma collected

by the American Red Cross during the war. The γ -globulins concentrated and isolated by the fractionation procedure have been found to contain 15 to 30 times greater concentration of antibodies than pooled serum reacting with diphtheria toxin, streptococcal erythrogenic toxin, influenza A virus, mumps virus, and the H antigen of *E. typhosa* (34). The γ -globulin fraction has been found to be of considerable usefulness in the attenuation or passive protection against measles (85, 111). The globulin fraction appears to be as effective as convalescent serum or placental extract against this disease of infancy.

5. Isoagglutinins

The euglobulin fraction designated as fraction III by the Harvard group (22, 25) is rich in isoagglutinins for the specific blood type substances A and B of the red corpuscles. To obtain type specific preparations, collection of indiscriminate pooled plasma is useless. Plasma has to be collected from the blood of the correct human types. Individuals of blood group A containing anti-B isoagglutinin in their plasma constitute about 40% of the American population. Consequently, preparation of the concentrated anti-B isoagglutinin from donors of the A type constitutes a simple problem. Anti-A isoagglutinin is much rarer, since only 10% of the population belong to the B blood group. This problem has been ingeniously solved by Melin (82), who proposed the combining of the bloods of type O (representing 45% of the American population) and type B. Type O blood plasma contains both anti-A and anti-B agglutinins. Moreover, it is more active than type B plasma toward cells containing the rare cells of sub-types A_2 and A_2B . To eliminate the anti-A activity of the O plasma, advantage is taken of the fact that when O and B blood are mixed the B cells absorb the anti-B activity virtually completely, leaving in the plasma only the anti-A isoagglutinin activity. By the above procedure pools of anti-A and anti-B isoagglutinins are obtained in approximately equal amounts from a typical American population.

Anti-Rh isoagglutinin for typing may be obtained from specially selected, sensitized Rh-negative donors.

6. In Blood Coagulation

Products useful for promotion of blood coagulation have been derived from blood plasma. Transfusion with blood plasma to supply elements lacking in hemophilia and in other hemorrhagic conditions has been a common medical practice.

Preparations of prothrombin and thrombin of high potency from

plasma have been obtained by adsorption and elution from hydroxides and silicates of magnesium and aluminum and by salting out procedures (31, 83, 102, 103, 104). In the Harvard fractionation scheme, prothrombin is concentrated in Fraction III-2. Prothrombin is not stable, and it is commonly converted to thrombin after its isolation. Purified thrombin has also been obtained, after first activating the prothrombin of the serum, by salting out procedures.

Thrombin is useful in the treatment of surface bleeding.

The fibrinogen and thrombin isolated in the human plasma protein fractionation has been employed to prepare fibrin clots, fibrin films, and fibrinogen plastics (38). Fibrinogen coagulum has found employment in the operative removal of renal calculi (25) by trapping small free stones at open operation. Preformed fibrin films are useful for surface films as a dressing for burns (52). Fibrin foam has been prepared as a hemostatic agent designed to combine the function of an absorbable tampon with thrombin activity (8). Fibrin foam has been employed in neurosurgical operations (5); fibrin films in neurosurgery in the repair of dural defects and in the prevention of meningocerebral adhesions (6).

The observation that purified prothrombin is activated more slowly than prothrombin in native plasma by thromboplastin and calcium ions led to the discovery of the accelerator globulin. The Ac-globulin increases not only the rate of activation but also the final thrombin yields. In blood plasma the Ac-globulin apparently is present as an inert protein or pro-enzyme. It is changed to the active form found in serum by thrombin. Serum Ac-globulin is considered to be the active catalyst of the interaction of prothrombin, thromboplastin, and calcium ions by Ware and Seegers (117). Concentrated preparations of serum Ac-globulin have been obtained from bovine serum and from defibrinated oxalated bovine plasma after activation with a trace of thrombin. The procedure consists of precipitating it along with prothrombin from acidified diluted plasma and then redissolving in oxalated saline. The Ac-globulin can be adsorbed on magnesium hydroxide and then eluted with carbon dioxide (116).

Fibrin clots commonly dissolve after a short interval. This lysis is induced by a proteolytic enzyme present in a pro-enzyme form in serum and which can be activated by shaking the serum with chloroform. It has been proposed to name the pro-enzyme plasminogen and the active form plasmin (19). The proteolytic activity induced by chloroform occurs in an euglobin fraction with a minimum solubility at around pH 5.2 (112).

The lytic activity of the blood serum also is activated by a kinase

produced by hemolytic streptococci and named streptokinase (18). The plasmin so formed is able not only to dissolve fibrin, but also to act on other proteins. Its action can be inhibited by crystalline trypsin inhibitor. In the Harvard scheme it is concentrated in Fraction III-2, 3.

III. HORMONES

1. Insulin

a. Diabetes

Insulin is the most important hormone preparation from a medical standpoint. Its medical importance is demonstrated by the following statistics: The incidence of diabetes in 1940 was 3.67 per 1,000 population, leading to a figure of over 500,000 diabetics in the United States. The incidence increases steadily with age and amounts to 11.8 for males and 20.9 for females per 1,000 population at age 60 years. The greatest incidence of diabetes is between the ages of 50 and 64 years. Over 50,000 persons become diabetic in the United States each year and this disease has mounted to eighth place among the leading causes of death.

The presence of insulin is indispensable for the normal metabolism of carbohydrates and fats in mammals. Diabetes results from an absolute or relative deficiency of insulin. The hormone is produced by the β -cells of the islets of Langerhans of the pancreas and is discharged into the general circulation. The stimulus that incites secretion of insulin, apparently, is the blood sugar level; a high blood sugar concentration stimulates the secretion, and low blood sugar inhibits it (107). Prolonged high blood sugar is believed to induce excess secretory activity on the part of the islet cells, with damage to the capacity of the cells to secrete, eventually leading to a state of hydropic degeneration.¹

The sequence of events in experimental diabetes is as follows (79): Following pancreatectomy in an animal like the dog the blood sugar rises rapidly and the stores of glycogen are soon depleted. When the blood sugar increases above a concentration of about 185 mg. % the renal threshold is exceeded and glucose is excreted in the urine. The drain of sugar by glycosuria causes an increased catabolism of protein and lipids. This loss is aggravated by the increase in basal metabolic rate resulting from increased oxidation of protein. The energy needs of the animal are not met by the food it ingests and,

¹ Degeneration of the islet cells may also be produced by alloxan. This offers a useful means of inducing diabetes in animals for experimental study.

consequently, there is marked loss of weight and depletion of the fat depots. The increased fat catabolism leads to overproduction of ketone bodies. The glycosuria and ketosis induce a train of consequences which soon prove fatal. Large amounts of water and sodium chloride are lost in the urine. This leads to a dehydration. The excreted ketone acids rob the body of its normal supply of buffer cations (Na^+ and K^+). While this tendency is combated by the excretion of large quantities of ammonia, this defense is insufficient and the acidosis and dehydration progresses to coma and death.

The activity of the pancreas is dependent on the functional activity of the anterior pituitary, the adrenal cortex, the thyroid and the liver. A very important interrelation is between pancreas and liver, since liver is the chief source of blood sugar, which is mainly derived from its glycogen store, and is the organ most readily available for conversion of glucose to glycogen under the influence of insulin. To trace the interrelations between pancreatic islet function and the above mentioned endocrine glands is outside the scope of this volume. The reader is referred to the following sources for this information (13, 79, 107).

b. Chemistry

Insulin is quickly inactivated by the proteinases of the pancreas and success in its isolation depends upon inhibition of protease activity. This is accomplished by adding alcohol and acid to fresh pancreas. Procedures for the isolation and crystallization of insulin are given in Chapter V.

Crystalline insulin exhibits the properties of a typical protein. The usual crystalline preparations contain 0.3 to 0.6% zinc, but Sahyun (97) has obtained preparations with as low a zinc content as 0.15%. Crystalline insulin has been found to be electrophoretically homogenous and to have a molecular weight of 46,000 (see Table I) (62). Analysis of insulin has yielded no evidence for the occurrence in the molecule of any constituent different from the known amino acids or other characteristic protein components. An outstanding chemical property of insulin is its high sulfur content (3.3%) which is virtually all present in the disulfide form. The integrity of the disulfide linkage is essential for the biological activity of insulin, which is lost upon reduction of the disulfide bonds or by treatment with alkali. The evidence at hand leads to the conclusion that the hypoglycemic activity of insulin is a specific property of the whole protein molecule.

One of the great advances made in adapting insulin for better

clinical use was the preparation of protamine insulin by Hagedorn and coworkers (49) and protamine zinc insulin by Scott and Fischer (101). By combining insulin with the protamine, salmine, and a trace of zinc there is obtained an insoluble insulin complex which is

TABLE I
PHYSICAL AND CHEMICAL CHARACTERISTICS OF
CERTAIN HORMONES (69)

Anterior Pituitary										
Hormone	Species	Molecular Weight	Iso-electric Point pH	Nitro-gen %	Sul-fur %	Cys-tine %	Methi-one %	Tyro-sine %	Trypto-phan %	Hex-ose %
Growth	ox	44,300	6.85	15.65	1.30	2.25	3.06	4.3	0.84	0.0
Adrenocortico-tropic (ACTH)	sheep	20,000	4.7	15.65	2.30	7.19	1.93	2.5	0.54	0.0
	swine	20,000	4.7-4.8	15.47	2.33					
Thyrotropic	ox	ca 10,000			1.0					
Lactogenic	sheep	26,500	5.73	15.86	1.79	3.1	4.31	4.5	1.3	0.0
	ox	26,500	5.73	16.50	2.0	3.4		5.7	1.3	0.0
Interstitial cell stimulating (Metakentrin)	sheep	40,000	4.60	14.2				4.5	1.0	4.5
	swine	90,000	7.45	14.93					3.8	2.8
Pancreas										
Insulin	ox	46,000	5.30-5.35		3.3	11.0		12.3	0.0	0.0

slowly absorbed and has a very much more prolonged hypoglycemic action. A recent preparation intermediate in its hypoglycemic action between unmodified and protamine zinc insulin is globin insulin.

c. Clinical use

The main purpose of insulin administration is to correct the defects in carbohydrate metabolism and its resulting sequelae in the diabetic individual. The most acutely dangerous of these are the effects of the acetoacetic and β -hydroxybutyric acids, resulting from the incomplete oxidation of the fatty acids, in depleting the basic cations and fluids of the body and in inducing a severe state of acidosis. It is the resulting dehydration and acidosis that lead to diabetic coma. The acetoacetic acid, it is suggested, is mainly responsible for the dyspnea, dimmed perception and loss of consciousness of diabetic coma. These effects are caused by a stimulation of the respiratory center and depression of the sensory centers of the brain. Excretion of sugar in the urine by the diabetic carries with it a large amount of water and inorganic constituents which also are an important factor in producing dehydration (10). More chronic effects resulting from long standing diabetes are arteriosclerosis,

particularly of the coronary arteries of the heart, gangrene, and diabetic retinitis and cataracts.

Insulin administered with glucose and isotonic saline is life saving in combating diabetic coma. Taken in proper dosage it prevents the onset of deleterious effects and permits the diabetic individual to lead a nearly normal life. At present protamine zinc insulin is employed virtually universally to control the chronic diabetic state and where a prolonged action is desirable. Unmodified insulin is used where an immediate response is important or to treat diabetic coma. One injection of the appropriate dosage of protamine-zinc-insulin per day usually suffices to prevent glycosuria in most diabetic individuals.

The potency of insulin is expressed in terms of an International Standard in which one unit is the amount which lowers the blood sugar of a normal 2 kg. rabbit fasted for 24 hours to a level of 45 mg. per 100 ml. blood within 5 hours. Crystalline insulin preparations have a potency of approximately 22 units per mg. Biological assay is also determined by the production of convulsions in mice (10).

The dosage of insulin used is one which will prevent development of ketosis and glycosuria. This dosage will, of course, depend greatly on the nature of the diet, activity, weight of the individual and other factors. A useful rule propounded by Peck (88) is *to give enough carbohydrate to the patient to protect the liver and give enough insulin to protect the remaining function of the islets of the pancreas.*

2. The Parathyroid Glands

a. Physiology

The parathyroids consist of four small glands anatomically contiguous with the thyroid. Commonly two of the glands are embedded in the thyroid lobes and two lie upon the surface.

The parathyroid glands' main function is in the control of the calcium metabolism of the body. Loss of parathyroid activity as a result of disease (ideopathic) or following surgical removal of the thyroid leads to a condition known as tetany.

Tetany is characterized by a hyperexcitability of the nervous system and can result from a number of causes. The severity of the attacks ranges from mild spasms of muscles, such as facial spasm or fanning of the hands and feet to frank tremors, spasm of the respiratory muscles, and generalized convulsions. The attacks are associated with rapid respiration, fever, and muscle pains.

Photographs of a dog used in the classic experiments of Collip (21) illustrating tetany after thyroparathyroidectomy and its relief by injection of parathyroid extract are reproduced in Fig. 1.



FIG. 1. Photograph of dog 59 days after thyroparathyroidectomy. (A) In state of tetany. (B) Complete recovery three hours after subcutaneous injection of 3 ml of parathyroid extract. Collip, J. B. (21).

The tetany is related to a rapid drop in the level of serum calcium (in man from about 10 mg. to 5 or 6 mg. per 100 ml. serum). In cases developing tetany following operation for goiter or removal of a parathyroid tumor the serum calcium seldom falls below 7 to 8 mg. per 100 ml. Relief of tetany can be secured by the administration of extracts of the parathyroid. This leads to an increase in the level of the serum calcium. Tetany also may develop in infants with rickets associated with a low serum calcium and in cases of ideopathic steatorrhea.

A low concentration of serum calcium does not invariably result in the condition of tetany nor is the serum calcium concentration invariably low in tetany. Rats reared on a diet very low in calcium,

but otherwise adequate, do not develop tetany or neuromuscular hyperexcitability although the concentration of their serum calcium drops to values of 5 or 6 mg. per 100 ml. (11). On the other hand, deficiency of magnesium leads to a condition of tetany indistinguishable from parathyroid tetany (45). In magnesium tetany the serum calcium level is not reduced. Neither is it reduced in the tetany observed in cases of alkalosis.

Hyperactivity of the parathyroid glands, due to tumors or to unknown causes, also has a deleterious effect. Hyperparathyroidism (*osteitis fibrosa cystica*) causes decalcification of the skeleton and resorption of bony tissue (both trabeculae and shaft), which are replaced by fibrous tissue with formation of cyst-like cavities. This condition is accompanied by bone pain and great susceptibility to bone fracture; chemically there is an elevation of the serum calcium up to values of 15 or even 20 mg. per 100 ml., a drop in the serum inorganic phosphorus concentration (down to 1–2 mg. per 100 ml.) and a great increase in the level of the alkaline serum phosphatase. Relief is possible by surgical treatment where the condition is due to a parathyroid tumor.

One of the main treatments of parathyroid tetany consists of the administration of large amounts of calcium salts and of parathyroid extract. Medication with parathyroid extract cannot be continued for long as there gradually develops a resistance to its action and loss of effectiveness after repeated use. The calcium level of the blood can also be increased by massive doses of vitamin D preparations or dehydrotachysterol and these also are used in the treatment of tetany (3).

Other therapeutic uses of parathyroid extract are dependent on its effect in decalcifying the body and increasing the blood level of calcium and the urinary excretion of phosphate and calcium. A decalcifying regimen is effective in removing abnormal metallic ions which may be stored in bone. Parathyroid extract and a low calcium diet has been employed for this purpose in the treatment of lead, radium and certain other metal ion poisoning.

b. Chemistry

Potent parathyroid extracts were prepared by Collip (21) by extracting the glands with hot 5% HCl, removing the fat which separated, and readjusting the solution with alkali to pH 8. Inert protein was removed by several isoelectric precipitations with dilute HCl and the residual filtrate contained the active principle. The most potent extracts have recently been prepared by defatting and

dehydrating the glands with acetone and chloroform, extracting the gland powder with warm 0.2 N HCl, fractionating the fat cleared extract with acetone (80% acetone by volume) and discarding the precipitate. The hormone is precipitated from the filtrate by adjusting the pH to about 7 and increasing the acetone concentration to 86% by volume. This procedure yields a material with an activity of about 100 units per gm. of fresh gland² (55).

The best preparations of the parathyroid hormone are admittedly impure. A molecular weight determination in the ultracentrifuge of a preparation containing inert protein indicated a value of between 15,000 and 25,000.

Test on a purified preparation by Ross and Wood (96) gave values of around 13% N and amino nitrogen between 6.5–7.0%. Molisch's test for carbohydrate was negative. The active material is soluble in water and in 80% alcohol or acetone. It is insoluble in ether or pyridine. The hormone is stable in dilute acid solution. It is quickly inactivated by pepsin and trypsin which explains why the hormone is ineffective when administered orally. It is also inactivated by acetylation with ketene (123).

3. Pituitary Hormones

The pituitary gland consists of two main parts, the *anterior* lobe and its accessory portions (*pars tuberalis* and *pars intermedia*) and the *posterior* lobe with the *pars nervosa*.

a. Posterior pituitary gland

The normal physiological function of the posterior pituitary gland is in doubt. Extracts of the gland have pharmacological effects on the heart, respiration, smooth muscle, and on salt and water metabolism.

Intravenously injected extracts produce an increase in blood pressure which is maintained for several minutes. The pressor effect is not antagonized by ergotoxin nor is it prevented by denervation. This indicates that the action is due to direct peripheral vasoconstriction. Posterior pituitary extracts also cause a constriction of the coronary blood flow. Successive injections made at intervals more

² The U.S.P. unit of parathyroid hormone activity is 0.01 of the amount of hormone required to raise the serum calcium of a 10 to 12 kilo dog by 1 mg. per 100 ml. within 16 hours after subcutaneous injection of the test material. Tepperman, L'Hereux and Wilhelmi (113) have observed that subcutaneous injection of parathyroid hormone in male albino rats produces a fall in serum inorganic phosphorus in three hours which is directly proportional to the logarithm of the dose of administered hormone. This relation can be used to establish a standard dose-response curve which is proposed for use in the standardization of parathyroid activity.

frequent than 15 minutes produce progressively diminishing blood pressure responses until a point is reached where the animal fails to respond entirely (tachyphylaxis). The respiratory changes are an increase in the respiratory rate interspersed with periods of cessation of breathing (59).

Extracts of the posterior lobe cause the contraction of smooth muscle (oxytotic effect), particularly of the uterus and the gastrointestinal tract.

Deficiency of the posterior pituitary induces a condition characterized by excessive water secretion known as diabetes insipidus. This disease is controlled by the administration of posterior pituitary preparations. In normal animals, administration of the extracts causes retention of water and loss of salt. The extracts are believed to act directly on the kidney to increase the tubular reabsorption of water and decrease the reabsorption of salt.

Fractionation of the posterior pituitary has yielded preparations with separate pressor and oxytotic activities. The pressor principle, is apparently also responsible for the antidiuretic action. The purest hormone fractions that have been obtained are polypeptides that have molecular weights between 600 and 2,000. The pressor principle contains the amino acids tyrosine, cystine, and arginine and probably proline and isoleucine. The oxytotic hormone contains tyrosine, cystine and arginine and probably proline and leucine (60).

The two fractions differ in electrical mobility, the pressor principle moving at a much more rapid rate in an electrical field than the oxytotic principle. The isoelectric points also are different, that of the pressor fraction being at pH 10.8, and of the oxytotic fraction at 8.5 (60).

The pressor, oxytotic and antidiuretic activity of purified hormone preparations are rapidly destroyed by trypsin, whereas pepsin has no effect upon any of them. The posterior pituitary hormones are quite stable in dilute acid of pH 3 and they can stand boiling at this pH for several minutes without loss of activity. On the other hand, they are quite unstable in alkaline solution.

Many investigators have maintained that the three distinct biological activities of the posterior lobe were properties of a single "mother-molecule" in the gland. Substantial proof for this belief has been obtained by Van Dyke and coworkers (115) who have isolated an apparently homogenous protein from the gland which possesses pressor, oxytotic, and antidiuretic activity.

Extracts of the posterior pituitary have important medical applications. The pressor effect is standardized by the increase in blood

pressure produced in the anesthetized cat or dog. The oxytocic effect is assayed by the contraction produced in an isolated strip of guinea-pig uterus. The antidiuretic effect is determined from the effect on the rate of urine excretion in the rat. In the best preparations of the hormone that have been reported, the potency of the pressor principle was 450 units per mg. and 700 units per mg. for the oxytocic hormone (59).

Posterior lobe extracts are used in obstetrics in the treatment of postpartum hemorrhage and to induce the onset of labor. The material carrying the pressor activity is successfully employed in the treatment of diabetes insipidus.

b. Anterior lobe

The anterior pituitary is often described as the master gland of the endocrine system of the body. Six fairly homogeneous proteins with distinct and individual hormone activities have been isolated from anterior pituitary extracts: namely, the growth hormone (somatotropic), the thyrotropic, the adrenocorticotropic, the lactogenic (prolactin) and the two gonadotropic hormones; follicle stimulating (FSH) or thylakentrin and the interstitial cell stimulating (ICSH) or metakentrin (17). Efforts to isolate distinctive hormone fractions for other physiological effects produced by the anterior pituitary such as its diabetogenic and its ketogenic activity have so far been unsuccessful (13).

The greatest triumphs of anterior pituitary research have been in the advancement of the understanding of the physiological functions of this gland and in the clarification of the etiology of certain endocrinological disorders. The therapeutic application of anterior pituitary preparations has made little progress and, in general, the results have been disappointing. A recent exception is the adrenocorticotropic hormone.

Destruction of the hypophysis leads to the following sequence of change: (a) arrested growth, (b) atrophy of the gonads and, indirectly, of the accessory sex glands, (c) suppression of milk secretion and involution of the mammary glands, (d) atrophy of the thyroid, (e) atrophy of the adrenal cortex, (f) a lowering of the metabolic rate, (g) hypoglycemia, an increased sensitivity to insulin and reduction in the content of liver and muscle glycogen, (h) depression of spontaneous activity, and (i) diminished resistance to infection and injury (13). A comparison of a hypophysectomized with a normal young rat is given in Fig. 2.

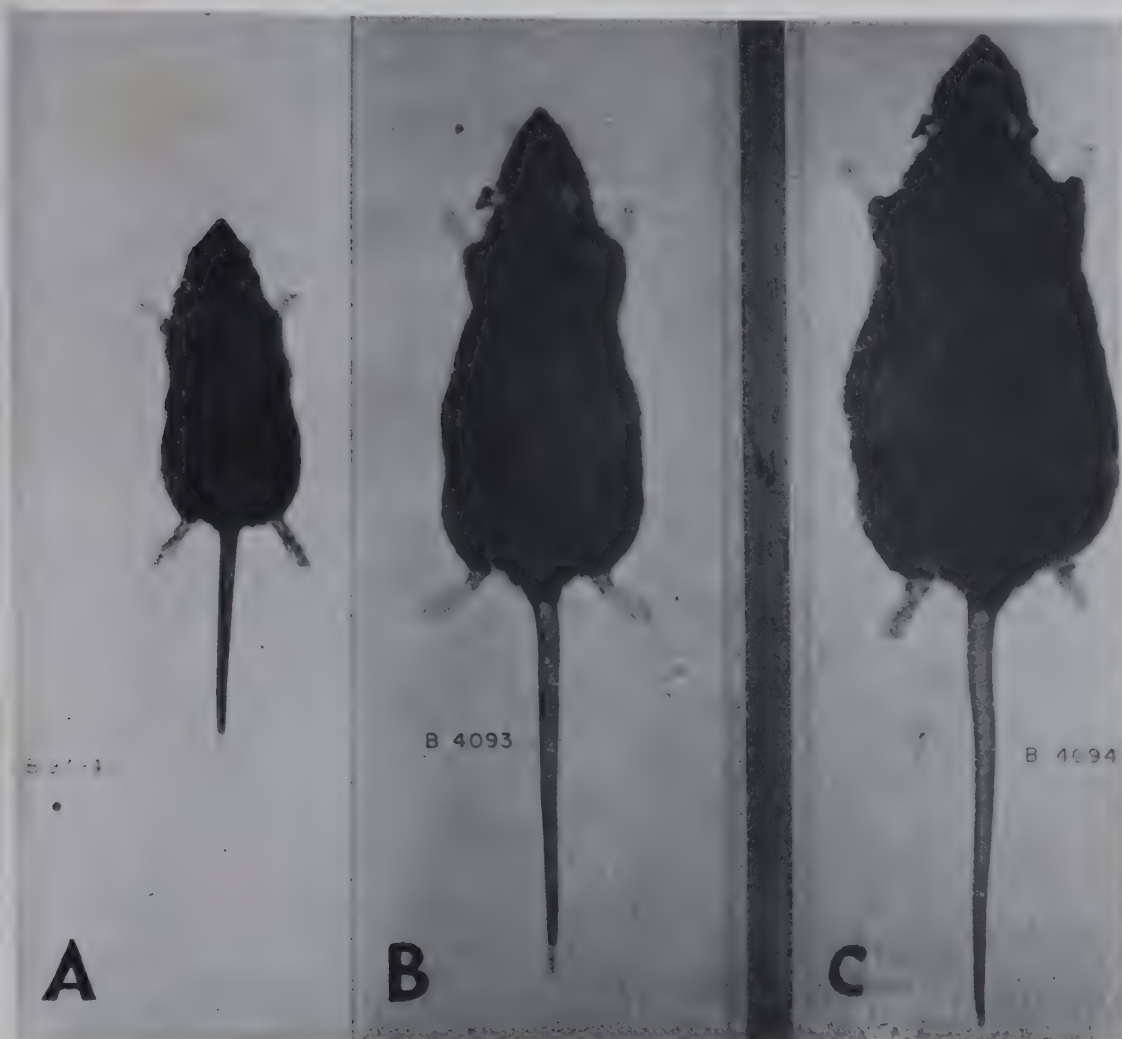


FIG. 2. Comparison of size of hypophysectomized, control and growth hormone treated female rats of the same age. (A) Hypophysectomized. (B) Control. (C) Treated with growth hormone for 435 days. Courtesy of Dr. C. H. Li and Dr. H. M. Evans, University of California.

In the rare disease hypophyseal cachexia (Simmond's disease), which involves failure of the function of the entire anterior pituitary, the changes listed above are found in man. The outlook in this disease is grave and death often follows a minor illness or injury. Attempts to treat Simmond's disease with pituitary extracts have yielded little success.

Certain types of dwarfism and infantilism are the result of disease processes involving failure of secretion of the growth and gonadotropic hormones of the anterior pituitary. Promising results in promoting growth in pituitary dwarfism have been obtained in a few selected cases with crude preparations of the growth hormone (35).

Excess production of growth hormone leads to the conditions of gigantism prior to the closure of the epiphysis and to acromegaly in

adults. Correction of the endocrine excess can only be obtained by means of surgery or x-ray treatment, not by endocrine therapy.

Gonadotropic preparations have been used with success in the treatment of undescended testes (105).

Biological assay of increased amounts of gonadotropins in the blood or urine of women is important for the determination of pregnancy.

1. **Growth hormone:** Growth hormone is a protein comparatively insoluble in water. The hormone activity is destroyed by pepsin and trypsin and heating to 100°. It is more stable in alkali than acid.

An amorphous preparation that is homogenous by electrophoretic and solubility tests, and is monodisperse in the ultracentrifuge, has been isolated by Li and coworkers (72). Crystalline products have been obtained by Wilhelmi and coworkers (41, 122a) and by Li, Evans and Simpson (74). Certain properties of growth hormone are given in Table I. Iodination and acetylation experiments indicate that the phenolic residue of tyrosine and certain of the free amino groups are essential for the biological activity of the hormone.

Stimulation, first of chondrogenesis and later of osteogenesis is perhaps the most characteristic biological effect of growth hormone.

Hypophysectomy decreased the thickness of the cartilage plate of the proximal end of the tibia—due to a disturbance in cartilage formation in the immature rat; administration of growth hormone reverses the regressive changes. The width of the uncalcified cartilage zone has been found to be proportional to the logarithm of the hormone dosage. This offers a more rapid and sensitive test than the previous one based on the resumption of growth in hypophysectomized young rats. By the growth method one growth hormone unit is the amount which will induce a total gain of 10 gm. in body weight in a 10 day period. The method based on the width of the cartilage plate is about three times as sensitive as the growth assay and can be used to detect as little as 10 micrograms of purified hormone (36, 71).

Growth hormone has a pronounced effect on nitrogen metabolism. It induces a retention of nitrogen and a reduction in the blood non-protein-nitrogen and urinary nitrogen. It produces an increase in protein and a decrease in the fat content of the bodily tissues (76). Its relation to bone metabolism is indicated by the fact that its administration to hypophysectomized rats causes an increase in the inorganic serum phosphorus concentration and the alkaline phosphatase of the plasma (70).

The striking effect of growth hormone on protein synthesis is

demonstrated by tracer experiments with labeled amino acids (42). In Table II it is shown that growth hormone produced a 70% increase in the incorporation of S³⁵-labeled methione in muscle.

TABLE II
INCORPORATION OF S³⁵ OF METHIONINE INTO SKELETAL
MUSCLE PROTEIN^a

Treatment	Weight Gain ^b g.	Specific Activity × 100 × Wt. of Animal in kg.
		Administered Dose S ³⁵
Normal mice	0.3 ± 0.1	0.034 ± 0.001 (6)
Growth hormone-treated mice	1.0 ± 0.1	0.046 ± 0.002 (6)
Hypophysectomized rats	-4.0 ± 0.9	0.20 ± 0.02 (2)
Growth hormone-treated hypophysectomized rats	14.0 ± 3.1	0.34 ± 0.04 (3)

^a Incorporation of methionine expressed in terms of a function of the specific activity S³⁵/S³². Data are arithmetic means ± standard error of mean. Value in parentheses indicates number of animals used.
^b Weight gains are for the period of hormone administration. Control and hormone-treated animals were of the same age and were selected to be of approximately the same weight at the time of administration of labeled methionine. Average weights were:
Normal mice 19.4 ± 0.2 g.
Growth hormone-treated mice 19.6 ± 0.2 g.
Hypophysectomized rats 190 ± 11.3 g.
Growth hormone-treated hypophysectomized rats 198 ± 8.2 g.
Friedberg, F., and Greenberg, D. M. (42).

In normal animals continued administration of high dosages produces gigantism. Li and coworkers (70) obtained continuous growth on 200 day old female rats for a period of 435 days with a gain of 289 gm. in weight, while the control untreated females gained but 57 gm. Photographs are shown in Fig. 2.

2. **Adrenocorticotropic hormone (ACTH):** This hormone acts upon the adrenal cortex causing it to hypertrophy. In the hypophysectomized animal it will restore the atrophied adrenal cortex to normal and in high levels will produce an abnormal hypertrophy. The maintenance in weight or increase in weight of the adrenal cortex in the hypophysectomized animal or the histological changes in the lipid picture is commonly used as a method of assay.³ As an example, in 40 day old hypophysectomized male rats the adrenal weight was maintained at 26 mg. by single daily injections of 0.2 mg. of purified hormone for 15 days while in the untreated controls the weight of the adrenals regressed to 12 mg.

³ A rapid and sensitive test for ACTH based on the depletion of the ascorbic acid content of the adrenals in hypophysectomized rats by this hormone has been developed by Sayers, Sayers and Woodbury (Endocrinology 42: 379 (1948)). The depletion is expressed as the difference between the concentration of ascorbic acid in the left adrenal, removed immediately before hormone injection, and the concentration of ascorbic acid in the right adrenal, removed one hour after the intravenous injection of hormone.

Preparations of ACTH consisting of a single protein component have been prepared by several investigators. The starting material is the acid-acetone extract first used for the preparation of lactogenic hormone by Lyons (81). La and coworkers (73) purified the hormone from sheep glands by a procedure which involves repeated fractionation from a solution of pH 3 first with 0.5 *M* NaCl, in which the hormone is soluble, followed by addition of NaCl to 1.35 *M* in which it is insoluble.

Sayers, White and Long (99) purified hog ACTH by dissolving the crude extract at pH 9 and successively lowering the pH to 8.0, 6.6 and 5.4. The hormone is then precipitated from a solution of pH 5.4 by adding $(\text{NH}_4)_2\text{SO}_4$ to 0.07 saturation and then adding 4 volumes of acetone. In the final step the material is dialyzed, the salt-free solution is adjusted to pH 5.4 the resulting precipitate is discarded, and the filtrate is lowered to pH 4.7. This precipitates the ACTH.

ACTH is an unusually stable protein. It will withstand heating in neutral solution at 100° for two hours and in 0.1 *M* HCl for one hour. Heating in 0.1 *M* NaOH for 30 minutes causes loss of activity. The hormone activity is not destroyed by the action of pepsin but is by trypsin. ACTH is very soluble in water but is precipitated by 2.5% trichloroacetic acid. The purified preparations contain no free —SH groups and by the tests of acetylation and iodination require the presence of free amino groups and the free phenolic residues of tyrosine for hormonal activity.

The action of ACTH is largely of an inhibitory character on the body physiology. This is because of the characteristic effects of the steroid hormones whose secretion it stimulates.⁴ It causes an inhibition of growth in the rat and retards bone formation by inhibiting chondrogenesis and osteogenesis. Associated with this is a lowering of the plasma alkaline phosphatase. It causes an involution of the thymus and the lymph nodes and dissolution of lymphocytes. Injection induces a blood lymphopenia. Its retarding effect on nitrogen metabolism is indicated by an increase in the urinary nitrogen excretion, loss of body weights, and increase in the content of liver arginase (71).

⁴ Of great medical interest is the recent observation that ACTH stimulates the adrenals to secrete steroids of the nature of cortison that are effective in causing a remission of the pathological disturbance in rheumatoid arthritis. La has reported (Fed. Proc. 8: 219 (1949)) and it has been confirmed by Brink, Messenger and Folkers (J. Am. Chem. Soc., 72: 1040 (1950)) that the hormone retained its adrenocorticotrophic activity and was active in rheumatoid arthritis after partial hydrolysis into peptide fragments with an average chain length of eight amino acid units.

3. Thyrotropic hormone: The thyrotropic hormone is assayed by its effect in converting the flattened cells of the thyroid of the hypophy-ectomized rat into columnar epithelium. A unit of the hormone is the amount necessary to convert the squamous epithelium into a low cuboidal structure with spherical rather than flattened nuclei.

An electrophoretically homogenous product has been obtained by Ciereszko (20). One gamma of this preparation gives a histological response in the thyroid of the chick.

The essence of the method of preparation consists of extracting ground frozen beef pituitaries with 2% NaCl at a low temperature and pH 7.8. The extract is then adjusted to pH 4.1 and the hormone is precipitated by fractional precipitation by addition of acetone to 75% by volume. Further purification is effected by adjusting the aqueous extract of the acetone precipitate to pH 9 and then adjusting to pH 7 and adding 5% lead acetate. Several inert precipitates are removed by these treatments. Trichloroacetic acid is next added to 0.5 *M* concentration, the precipitate that forms is removed and the resulting solution is dialyzed and concentrated.

Thyrotropic hormone appears to be a small protein molecule. Its reported molecular weight is only about 10,000, although it is not dialyzable. The characteristic physiological effects of the thyrotropic hormone are those resulting from its stimulation of the activity of the thyroid.

4. Gonadotropic hormones: The gonadotropic hormones of the anterior pituitary are considered to be the follicle stimulating hormone (FSH, thy lakentrin) and the interstitial cell stimulating hormone (ICSH), metakentrin). In addition, the lactogenic hormone also has a leuteotropic effect. The effects of the gonadotropic factors on the ovaries have been summarized by Fevold (39) as follows:

1. Non-antral containing follicles + thy lakentrin = macroscopic follicles.
2. Macroscopic follicles + thy lakentrin + metakentrin = secreting follicles.
3. Secreting follicles + thy lakentrin + metakentrin = preovulatory swelling and ovulation.
4. Mature follicles + thy lakentrin = corpora lutea.
5. Corpora lutea + prolactin = secreting corpora lutea.

In the male, thy lakentrin stimulates the development of the seminiferous tubules of the testes and metakentrin induces the production of androgen by the testicular interstitial tissue.

In addition, other well characterized gonadotropins are chorionic gonadotropin, produced initially by the fertilized ovum and subsequently by the placenta, and equine gonadotropin present in the blood serum of the pregnant mare (48). Chorionic gonadotropin is extensively excreted in the urine while equine gonadotropin is not.

A chemical characteristic of the gonadotropins is that they are glycoproteins. Human chorionic gonadotropin contains 16–17% reducing sugar (10.7% galactose, 5.2% hexosamine) equine gonadotropin about 25% (17.6% galactose, 8.4% hexosamine).

Thylakentrin has been isolated free from other hormones but is not homogenous by electrophoretic or ultracentrifugal analysis. Preparations of metakentrin have been prepared that meet the established criteria for the purity of a protein. Some properties of this hormone are recorded in Table I. Distinctive chemical differences are found with the same hormone from different sources. Thus, sheep metakentrin has a molecular weight of 40,000, isoelectric point at pH 4.6, and a content of 4.5% carbohydrate and 1% tryptophan, while hog metakentrin has a molecular weight of 90,000, isoelectric point at pH 7.45, and a content of 2.0% carbohydrate and 3.8% tryptophan. Hog metakentrin is insoluble in $\frac{1}{2}$ saturated ammonium sulfate, while thylakentrin is soluble even in $\frac{1}{2}$ saturated solution. The two gonadotropins are both inactivated by ketene and by cysteine. This indicates that three amino groups and the disulfide linkages are essential for the hormonal activities of both gonadotropins.

5. Lactogenic hormone (Prolactin): In the mammary gland, previously acted upon by estrogen and progesterone, prolactin induces the secretion of milk. A very sensitive test for prolactin which is used in its assay is the stimulation of the development of the crop gland in the pigeon.

Purification of prolactin is accomplished through the fact that it is soluble in acid-acetone at pH 1.5 and is precipitated upon increase in the acetone concentration to 92% (75, 81). The isolated prolactin can be crystallized from an acetic acid-pyridine solution or by precipitation from dilute acetone solution (121).

Characteristic physical and chemical properties of prolactin are given in Table I.

Prolactin is relatively heat stable and can be boiled for 15 minutes at pH 1 to 9 without loss of activity. Loss of biological activity occurs upon destruction or substitution of the free amino groups or phenolic group of tyrosine and also upon reduction of the prolactin with excess cysteine or thioglycolic acid (119, 120).

4. Miscellaneous Hormones

a. Thyroglobulin

The characteristic hormonal effects of the thyroid can be produced by administration of thyroxine or of crude thyroid substance orally. However, there are good reasons for believing that the true thyroid hormone is the thyroxine containing protein, iodothyroglobulin. This protein has been prepared in homogeneous form although the iodine content may vary from almost nothing up to 1.7% (122). The molecular weight has been determined as 675,000 (98). Upon hydrolysis there is obtained the common amino acids and also small amounts of thyroxine and diiodotyrosine. Upon enzymatic digestion there is obtained a fraction very resistant to peptidase action which consists of a mixture of thyroxine and thyroxine containing peptides.

The amino acid distribution in the thyroglobulin molecule, with the exception of tyrosine, diiodotyrosine and thyroxine, has been found to be the same in the normal, colloid, or adenomatous gland (15). Thyroglobulin from the glands of patients with colloid goiter has a low content of iodine-containing amino acid residues and is correspondingly higher in tyrosine content. The thyroglobulin of hyperthyroid subjects treated with lugols solution shows a greatly increased diiodotyrosine content, and a much lesser increase in thyroxine.

From the work of Cavett, *et al.* (16) it is indicated that the tyrosine residues present in thyroglobulin are capable of conversion to diiodotyrosine and thyroxine without breakdown of the protein. This offers no difficulty in the case of diiodotyrosine as this transformation can readily be achieved in all tyrosine containing proteins by iodination.

The formation of thyroxine in the intact protein also can no longer be doubted as it has been shown that thyroxine is formed in a variety of proteins by incubating them with iodine at 37 to 70° in slightly alkaline solution, particularly in the presence of an oxidizing agent such as H_2O_2 or Mn_3O_4 (78, 95). A similar enzyme catalyzed reaction is envisioned in the formation of thyroxine from tyrosine in the thyroid gland.

b. Secretin

The historical development of hormones is closely associated with secretin. The action of this substance in stimulating the flow of pancreatic juice and bile was demonstrated in 1902 by Bayliss

and Starling. The term *hormone* was invented by them to express the characteristic stimulating action of secretin.

Two laboratories have reported the isolation of secretin as a crystalline picrolonate. Hammersten and coworkers (50, 51) obtained a protein-like material with only a small proportion of picrolonic acid; Greengard and Ivy (47) obtained a product consisting of 80% picrolonate and what is assumed to be a peptide. Medically, secretin has found use in the diagnosis of pancreatic disease. The status of other hormones of the gastrointestinal tract is discussed in a review of Greengard (46).

IV. BACTERIAL TOXINS

Certain bacteria produce poisonous substances which are antigenic. These are called toxins. These are of two types, *exotoxins* in which the active material appears in the culture medium during growth of the microorganism and remains in solution after the organisms are removed, and *endotoxins* which are formed as intracellular constituents of the bacterial organism and cannot be freed without disintegrating the bacteria. Endotoxins are probably carbohydrate-lipid complexes.

The exotoxins are generally better antigens, are more toxic, are in general heat labile and sensitive to acid and alkali. The evidence is strong that they are proteins.

A number of bacterial toxins have now been isolated in highly purified form and have been shown to be proteins. No unusual constituents have been found in them so the toxicity apparently is characteristic of the molecule as a whole and is not dependent on some prosthetic group. This is confirmed by the ease with which the toxicity is destroyed by denaturation or by the action of formaldehyde.

Toxins are good antigens and produce antibodies upon injection into an animal which neutralizes the toxic effects. Immunization by direct injection is hazardous, so it is very fortunate that in a number of important instances (diphtheria, tetanus) the attenuated toxoids provide a satisfactory antigenic substitute.

The toxicity of a toxin can be neutralized by its antitoxin. The antibody has been found to combine in varying proportion depending on the ratio in which toxin and antitoxin are mixed. This neutralization does not permanently change the toxin as it is possible in some instances to recover the toxin by destroying the antitoxin, or in some cases the neutral mixtures can be activated by dilution.

Antitoxins flocculate toxins in the test tube as well as neutralize their toxicity, when mixed in proper proportions.

Snake venom, spider venom, and the poisonous vegetable proteins, ricin, crotin, and abrin resemble the bacterial toxins in their behavior and are classed among the toxins. Toxins from the venoms of the rattlesnake (named crotoxin) and from the cobra have been crystallized by Slotta and Fraenkel-Conrat (106) and by De (24a), respectively. Crotoxin is a homogenous protein of molecular weight 30,000. The toxicity and hemolytic effect of these toxins presumably depend on their lecithinase activity. Ricin, which is the highly toxic hemagglutinating protein of the castor bean has been obtained in crystalline form by fractionation with sodium sulfate (63). The isolated protein appeared to be a pure compound on the basis of electrophoretic, ultracentrifugal and immunological tests. However, the crystals were found to be 50% more toxic than the equally homogenous amorphous product.

Bacterial toxins that have been prepared in fairly pure form are diphtheria toxin (29, 87), streptolysin-O (53), the scarlet fever (erythrogenic) toxin (65, 110), botulinus toxin (1, 66), and tetanus toxin (90, 92, 93).

Success in the isolation of diphtheria toxin depended upon the development of a media giving good growth which contained no substances of greater complexity than an amino acid hydrolysate.

In the simplest procedure for purifying diphtheria toxin, the active material is salted out by saturated $(\text{NH}_4)_2\text{SO}_4$, and then fractionated by alternating between adsorption of impurities on $\text{Al}(\text{OH})_3$ cream and fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ (87). The purified diphtheria toxin contained 10^7 MLD⁵ mouse units per gm. of protein (87). Its isoelectric point was found to be at pH 4.1, the molecular weight 74,000 and dissymmetry coefficient 1.22 (89). Chemical data are 16.0% N, 0.75% S, 1.0% amino N, 9.5% tyrosine, 31.8% arginine and 5.3% lysine.

Certain strains of hemolytic streptococci grown in serum free media produce a filterable hemolysin which is reversibly inactivated by air and oxidizing agents and activated by certain reducing agents. The same organisms grown in a serum containing media produce hemolysin whose activity is not affected by oxidation-reduction. Todd (114) has designated the former as streptolysin-O and the latter as streptolysin-S. The two substances are immunologically distinct.

⁵ MLD = minimal lethal dose.

Streptolysin-O has been highly purified by Herbert and Todd (53) by a procedure which involves precipitation with saturated $(\text{NH}_4)_2\text{SO}_4$, fractionation with $(\text{NH}_4)_2\text{SO}_4$, adsorption and elution from $\text{Ca}_3(\text{PO}_4)_2$ gel and adsorption and elution from alumina C γ . The resulting product is a white powder readily soluble in water and dilute salt solution. Fairly concentrated solutions are stable for several weeks at 0° while dilute solutions undergo destruction.

The effect of hemolytic activity is maximal at pH 6.5 and the rate of hemolysis increases with temperature up to 38°. A reducing agent such as $\text{Na}_2\text{S}_2\text{O}_3$ or thioglycolic acid is required to secure all but a small amount of hemolytic activity. Maximal activation is given by all thiol compounds and $\text{Na}_2\text{S}_2\text{O}_3$; cyanide activates only feebly. Although activity is lost upon oxidation, 0.02 *M* iodoacetate produced no inhibition after 20 minute incubation at room temperature and only 22% at 38°. Iodacetamide, on the other hand, caused 60% inhibition under the latter conditions. Peculiarities of this nature in the activation-inhibition reactions of proteins are by no means uncommon. The antibodies formed by streptolysin-O injection are also capable of neutralizing tetanolysin and pneumolysin.

The scarlet fever (erythrogenic) toxin has been concentrated to a potency of 200×10^6 skin test doses per mg. of protein by adsorption on Lloyd's reagent and salting out with $(\text{NH}_4)_2\text{SO}_4$. This preparation exhibited five components upon electrophoresis in the Tiselius apparatus and was assumed to be about 50% pure. Further fractionation was achieved by electrophoretic separation, the toxin activity being present in the slowest migrating component. Although an electrophoretically pure product with a molecular weight of about 23,500 was obtained in this manner, it resulted in a decrease in the unit value of the toxin (65).

The toxin of *clostridium botulinum*, type A has been obtained in crystalline form by several groups of workers. Lamanna and co-workers (66, 12) isolated it from cultures grown on media containing casein, glucose and corn steep liquor. After acid precipitation at pH 3.5 the toxin crystallizes from 0.1–0.3 saturated $(\text{NH}_4)_2\text{SO}_4$ and controlled pH at the temperature of 4° in needle-like crystals. The procedure of Abrams, Kegeles and Hottle (1) differs from the above in that the acid precipitation is followed by salting out with 0.4 saturated $(\text{NH}_4)_2\text{SO}_4$. Crystallization is secured by dialysis at 4° of solutions containing at least 1% protein against 0.3 saturated $(\text{NH}_4)_2\text{SO}_4$ buffered at pH 6.8.

Evidence for the purity of this toxin are its constant activity,

which after two crystallizations remains 22×10^7 mouse MLD units per mg. of protein N, and its electrophoretic homogeneity in the pH range 3.2–7.0. That it is not completely homogeneous is indicated by the fact that it exhibits boundary spreading upon sedimentation in the ultracentrifuge (94).

The botulinus toxin is a globulin protein of 14.1% N content. It has its isoelectric point at pH 5.6 and its molecular weight, calculated from sedimentation and diffusion data, is 900,000 (94).

Tetanal toxin (spasmin) has been isolated and crystallized by Pillemer and coworkers (92, 93) with an activity of 6.6×10^7 mouse MLD units per mg. N.

The isolation was accomplished by fractional precipitation with methanol under controlled conditions of pH, ionic strength, protein concentration and temperature. Methanol was found to be preferable to ethanol because the inherent danger of denaturation by increased temperature is less for methanol. Certain proteins which are denatured by ethanol at -5° are quite stable in methanol even at 0° . The toxin, after preliminary purification, was adjusted to a 1% concentration and crystallized from 20% methanol at pH 6.0, μ 0.02 and temperature of -5° . Traces of nucleoprotein interfere with crystallization. The crystals disintegrate rapidly at temperatures above -5° and dissolve instantly in 0.15 *M* acetate buffer of pH 6.5. The toxin is very stable at 0° in the presence of 0.3 *M* glycine between pH 5–6. The crystalline tetanal toxin exhibited a single electrophoretic boundary and an almost constant phase rule solubility curve; evidence that it is a reasonable pure molecular species. Analysis of the toxin yielded values of 15.7% N, 1.04% S and the absence of carbohydrate. The isoelectric point is at pH 5.1 ± 0.1 .

Pillemer, Toll and Badger (91) isolated purified tetanal toxoid and have suggested that this is a dimer of tetanal toxin molecules which have apparently interacted or condensed through their toxic groups.

V. PEPTIDE ANTIBIOTICS

Antibiotics are chemical substances produced by living organisms which exhibit antimicrobial activity in comparatively low concentration. The antibacterial activity of antibiotics may be bacteriocidal but more commonly is bacteriostatic. The antibiotics of greatest potency and importance have been found among the fungi, with bacteria ranking next in importance. The contribution of the algae and higher plants to date has been negligible.

Gramicidin and tyrocidine: The polypeptides, gramicidin and tyrocidine have been of signal importance in the historical development of the rapidly expanding field of antibiotics. Although observations on antagonistic relations among microorganisms date back over 60 years, the first antibiotic to be isolated was tyrothricin, from cultures of *B. brevis* by Dubos in 1939 (28). This was later resolved into the two crystalline, alcohol-soluble polypeptides, gramicidin and tyrocidine. The classic studies of Fleming upon penicillin, from *penicillium notatum* appeared in 1929, but it was not until 1940, following the work of Dubos, that the intensive research program was initiated which led to the isolation and characterization of the peerless antibiotic, penicillin.

The antibiotics exhibit analogous effects upon microorganisms but are highly diverse in their chemical characteristics. They include α , β -unsaturated lactones, quinones, polypeptides and low molecular weight proteins. The latter are the ones that will be discussed in this section. The list of antibiotics that are peptides, their source and their range of activity are given in Table III. It is noteworthy that all the known peptide antibiotics are produced by bacteria and none by fungi. Tyrothricin is obtained by adjusting filtrates of autolyzed cultures of *B. brevis* to pH 4.8 and extracting the resulting precipitate with alcohol and then adding NaCl to salt out the tyrothricin. The product is an amorphous material of high antibacterial activity.

TABLE III (43)

Antibiotic	Produced by	Range of Activity
Diplococcin Gramicidin Gramicidin-S	<i>Streptococcus</i> sp. <i>Bacillus brevis</i> <i>Bacillus</i> sp.	Gram-positive cocci Gram-positive organisms Gram-positive and Gram-negative organisms
Subtilin Tyrocidine	<i>Bacillus subtilis</i> <i>Bacillus brevis</i>	Gram-positive organisms Gram-negative organisms

The individual components are obtained by treating the tyrothricin with a mixture of acetone and ether which dissolves gramicidin. The solvent is evaporated off and the pure gramicidin is obtained by recrystallization from acetone. From the acetone-ether insoluble fraction, the tyrocidine can be obtained by dissolving it in alcohol and crystallizing out with gaseous HCl. Gramicidin crystallizes as platelets of lenticular outline from acetone or dioxane. Its nitrogen content is 13.9%, it melts at 228–230° and has an optical rotation in alcohol of $(\alpha)_D = +5^\circ$. It gives a positive biuret

test and is negative for other protein reactions. The estimation of the molecular weight by isothermal distillation in methanol and butanol gave values of between 2500–4500. There are no free carboxyl or amino groups, which is evidence for the cyclic nature of the polypeptide. Gramicidin is unique in its high content of tryptophan and also in that the tryptophan is stable to acid hydrolysis. Hotchkiss (58), assuming a molecular weight of 2800, has suggested that gramicidin consists of six tryptophan residues, six of leucine, four each of valine and alanine, two of glycine and two of an unknown aminohydroxy compound. Of particular interest is the observation that gramicidin contains D-amino acids; nearly 50% of the α -amino nitrogen of gramicidin hydrolysates can be oxidized by D-amino acid oxidase.

The presence of the "unnatural" isomers has been confirmed by the isolation of D-leucine and of DL-valine; tryptophan and alanine were obtained in the L forms. Pure gramicidin can be bacteriostatic (and to some degree bactericidal) for Gram-positive cocci at levels of 0.01 to 1 μ gm. per ml. The action of gramicidin is little affected by peptones, pure proteins, carbohydrates, sterols or fatty acids. It is inhibited by phosphatides. A serious drawback in the use of gramicidin for the treatment of infections is that the material has to be applied directly to the site of infection and is chiefly protective rather than curative. Another serious drawback is its high toxicity to the host as 2 mg./kg./day is a fatal dose for a rabbit or a dog.

Topical application in the treatment of localized infections, wounds, or ulcers is the chief application. Tyrothricin, being more available than gramicidin is the form generally employed.

Tyrocidine contains 14.3% N, crystallizes in the form of needles and has an optical rotation in alcohol of $(\alpha)_D = -101^\circ$. It too is a cyclic peptide of molecular weight between 1,000–3,000. Hotchkiss considers it is a molecule of 26 nitrogen atoms, containing three phenylalanine and three ammonia residues and two residues each of glutamic acid, aspartic acid, ornithine, tryptophan, tyrosine, proline, leucine, and valine. The phenylalanine of tyrocidine is reported to be the D-isomer; the other amino acids of the L-form.

Tyrocidine is effective in killing Gram-positive and Gram-negative bacteria only in the absence of serum or proteins or peptones and consequently has little activity *in vivo*. Like gramicidin, it is highly toxic to animals.

Little can be suggested concerning the mode of action of these antibiotics. The D-configuration of certain amino acids probably

confers an enhanced stability to these compounds which is bacteriostatic for other reasons. Gramicidin increased the respiratory rate of susceptible organisms in which glucose is the substrate; tyrocidine, on the contrary, decreases the respiratory activity of susceptible bacteria. With lactic acid as substrate, gramicidin has no effect on its oxidation; the oxidation of glycerol is inhibited. The presence of gramicidin prevents the conversion of inorganic phosphate of the medium by staphylococci respiring in glucose to organic phosphates. Hotchkiss considers that this effect of gramicidin upon phosphate uptake is more closely related to its bacteriostatic effect than is the effect upon oxygen uptake.

Tyrocidine, by virtue of its basic groups, is a bactericidal cationic detergent, unique only in its natural origin and complexity of chemical composition. It produces lysis and autolysis of susceptible bacteria and causes the release of soluble cell constituents. Apparently, the bacteriolysis by tyrocidine is a secondary phenomenon, following slowly upon a rapid primary injury of the bacterial surface. Upon acetylation of tyrocidine, there is eliminated both its bacteriocidal activity and its effect on cell surfaces. Various cationic and anionic detergents (Duponol C, hexoresorcinol) give about the same effect as tyrocidine, in about the same amounts (58).

Gramicidin-S: This antibiotic was isolated in 1944 and because of the similarity of the source to *B. brevis*, it was named "Soviet gramicidin" or gramicidin-S (44). The antibiotic is isolated by acidifying the culture medium and extracting the resulting precipitate with alcohol. Gramicidin-S precipitates in crystalline form upon evaporating the solvent. It is active against both Gram-positive and Gram-negative bacteria. Gramicidin-S contains 13% nitrogen, melts at 268–270° and has a molecular weight of 1060–1340. Fourteen percent of its total nitrogen is in free amino groups. Amino acids that have been identified are ornithine, proline and leucine; lacking are tryptophan, tyrosine, phenylalanine, arginine, histidine, glutamic acid and aspartic acid.

Diplococcin was isolated from cultures of a species of streptococcus by Oxford (86). It is precipitated as the picrate, and this is then decomposed with acetic acid. This antibiotic appears to be a low molecular weight protein containing 14% N but no sulfur or phosphorus. Positive color tests are given for arginine, tyrosine, tryptophan, and the Molisch carbohydrate reaction. Its antibiotic activity is against Gram-positive cocci.

Subtilin, produced by a strain of *B. subtilis*, is a basic poly-

peptide in which the free amino groups outnumber the free carboxyl groups and which diffuses only slowly through cellophane membranes (27). It has been obtained in an electrophoretically homogenous form and with a constant bacteriostatic potency and nitrogen and sulfur content (40). The nitrogen content is 14%; the sulfur 4.2%. Chemically, subtilin is unique in that all its sulfur is derived from the amino acid lanthionine—not cystine or methionine. The rest of its makeup consists of the usual L-amino acids found in proteins. Absent, in addition to the above-mentioned sulfur-containing acids, are serine, threonine, tyrosine, arginine and histidine. Subtilin contains 15.8% nitrogen, 1.8% free amino nitrogen, and 4.8% sulfur. Analysis by Lewis and Alderton (68) yielded 4.5% glycine, 12% alanine, 3.2% L-valine, 4.2% L-isoleucine, 14.7% L-leucine, 4.8% L-phenylalanine, 3.3% L-proline, 6.0% tryptophan, 10.8% L-lysine, 4.0% aspartic acid and 11.8% L-glutamic acid. The amino acid data suggest a molecular weight of about 7,000.

Subtilin is soluble in acidified water, aqueous alcohols, acetic acid and formamide. It is insoluble in salt solutions, anhydrous alcohols, acetone, and lipid solvents. It is inactivated by alkali, strong acid and proteolytic enzymes.

In neutral solution the solubility of subtilin is about 2%. Sodium chloride even to the extent of a few hundredths of a percent reduces the solubility greatly. In 0.85% NaCl solution and pH 7.4 the solubility is only 0.06–0.07%. Ten percent NaCl solution precipitates subtilin quantitatively. The low salt solubility of subtilin is used to advantage in its purification. In the presence of salt about 90% of the subtilin is extracted with half a volume of *n*-butanol. Upon separation of the butanol extract, excess solid salt is added which dehydrates the butanol to give the two liquid phases, dry butanol and salt-saturated water. Subtilin is insoluble in either phase and may be collected by centrifugation. The subtilin is taken up in 0.4% NaCl solution at pH 4.6 and is then precipitated by increasing the salt concentration to 10% (77).

The low solubility of subtilin in salt, however, seriously handicaps its use for parenteral administration. The antibiotic precipitates locally on subcutaneous or intramuscular injection. Intravenous injection must be carried out very slowly to avoid precipitation in the blood stream.

To overcome this objection experiments have been undertaken to modify the subtilin so as to increase its solubility but still retain its bacteriostatic action. Substitution of the free amino groups

was found to cause loss of biological activity. Esterification of the carboxyl groups, on the other hand, by reaction in aqueous or anhydrous methanol or ethylene glycol in the presence of HCl as a catalyst, has yielded a product with doubled solubility and with enhanced *in vitro* bacteriostatic activity (14).

VI. VIRUSES

1. Animal and Plant Viruses

Many reasons can be cited for the lively scientific interest in the viruses. First and foremost, they are responsible for some of the most devastating diseases of man, animals and plants. Virus-induced diseases of man include smallpox, yellow fever, dengue fever, poliomyelitis, influenza, measles, mumps, virus pneumonia, certain types of encephalitis, and the common cold. Virus diseases afflicting animals are hog cholera, cattle plague, foot-and-mouth disease of cattle, rabies and distemper in dogs, horse encephalitis, fowl pox, Newcastle disease of chickens, and certain benign and malignant tumors of rabbits and mice. Examples of virus diseases of plants are tobacco mosaic, peach yellows, aster yellows, potato yellow dwarf, alfalfa mosaic, tomato bushy stunt, and curly top of sugar beets.

Virus diseases in man exact an enormous toll in loss of life, in the suffering of the victims, and in impaired health; virus diseases in plants and animals are responsible for enormous economic losses. These diseases, therefore, present an ever present challenge to the scientist to develop methods of immunization against and for effective therapeutic agents for the treatment of virus diseases of men and animals and also other means of combating its ravages in plants and farm animals.

Another cogent scientific reason for the great interest in viruses is that they appear to bridge the gap between the protein molecules of the biochemist and living organisms of the biologist. Thus, study of their properties may be expected to throw light on the genesis and nature of life.

The term virus is used to designate those infectious agents capable of passing through filters that retain ordinary bacteria. As a group, the viruses are smaller than bacteria and it has not been possible to culture them on synthetic media. Viruses grow or reproduce only within living cells, and, in addition, in general, a given virus will multiply only within certain cells of a limited number

of animal or plant species. For example, the influenza virus proliferates only in certain cells of man and of a few animal species; growth of cucumber viruses 3 and 4 is confined to plants of the cucurbit family. An exception is tobacco mosaic virus which is capable of infecting over 50 plant species. In this instance the specificity probably is for a particular cell type. Another characteristic of singular importance is that during multiplication a virus may dramatically undergo a sudden and discontinuous change analogous to a gene mutation, which produces a transformation in the character of the specific disease induced by the virus. By such changes the virus can become more virulent or less virulent. Presumably the formation of a new virulent mutant strain of a virus may lead to an epidemic of the disease which may result in widespread death and suffering. The influenza pandemic of 1918 may be an example of a mutation to a particularly virulent virus strain. On the other hand, if the resulting strain is much less virulent it may be used to confer immunity by being used as a vaccine.

It should be emphasized that viruses were first recognized and continue to be recognized mainly through their biological activity, *i.e.*, the disease they produce.

Little definite could be said about the nature of viruses until 1935, when Stanley (108) crystallized a high molecular weight protein from infected tobacco leaves which was found to possess the properties of tobacco mosaic virus. Shortly afterward there followed the isolation of similar high molecular weight nucleoproteins which upon inoculation produced the characteristic virus diseases of their source materials. Examples are the viruses of aucuba mosaic, enation mosaic, tobacco ring spot, latent mosaic of potato, severe itch, Shope rabbit papilloma, bushy stunt of tomato, cucumber mosaic 3 and 4, tobacco necrosis, and the staphylococcus bacteriophage.

Isolation of viruses usually is accomplished by procedures involving high speed centrifugation. The reasons for this are that their large sizes makes separation by differential centrifugation comparatively simple and that this is a much milder procedure than separation by chemical means. Usually there is a great deal of loss of virus activity when isolation is attempted by chemical procedures.

Evidence on the size and shape of viruses has been secured from measurement of the sedimentation and diffusion constants, x-ray, light scattering, and other physical properties; most pre-eminently,

however, by means of the electron microscope. The viruses that have been isolated range in size from about 10 $m\mu$ to 300 $m\mu$. The smallest viruses (*e.g.*, alfalfa mosaic virus) are smaller than certain accepted protein molecules (Buscyon hemocyanin), while certain large viruses (*e.g.*, vaccine virus) are larger than certain accepted microorganisms (pleuro-pneumonia group).

Tobacco mosaic virus particles were found to consist of about 94% protein and 6% of ribose nucleic acid combined in a firmer bond than the usual salt linkage common in nucleoproteins. No differences in properties have been found in the virus isolated from different hosts or at different times of the year. The single particles of this virus are rod-shaped, of about 280 $m\mu$ in length and 15 $m\mu$ in diameter. The calculated molecular particle weight is about 40 million. There is good x-ray spectrographic evidence that a single virus particle is built up of similar subunits fitted together in a hexagonal lattice (9). The nucleic acid of the final structure appears to exist in the form of eight thread-like units laid down along the length of the particle. These single virus particles can aggregate in a two-dimensional pattern to form large needle-like crystals that are readily visible with a low-power hand lens. Of particular interest and significance is the fact that the tobacco mosaic virus particles contain no water and exhibit no enzymatic or metabolic activity other than virus activity.

Besides the rod-shaped anhydrous group of viruses represented by tobacco mosaic, a larger group appears to be essentially spherical in shape and hydrated. Some examples are alfalfa mosaic (diameter 17 $m\mu$), tobacco ringspot (diameter 19 $m\mu$), tomato bushy stunt (diameter 26 $m\mu$), rabbit papilloma (diameter 40 $m\mu$), influenza (diameter 100 $m\mu$) and vaccine virus (diameter 225 $m\mu$). Of these, only tomato bushy stunt virus has been obtained in crystalline form. It crystallizes in the form of large rhombic dodecahedra which are made up of protein molecules that appear to be strictly homogenous with respect to size, shape and density. The estimated molecular weight is only 8 to 9 million. This virus consists of about 17% nucleic acid and 83% protein. The structural complexity of the bushy stunt virus nucleoprotein is but little more complex than that of hemoglobin and no more complex than that of hemocyanin. The composition of the influenza virus consists of about 60% water, with the solid portion composed of about 65% protein, 25% lipid, 7% carbohydrate, and a very small amount of nucleic acid. Vaccine virus, which is the largest and most complex

virus subjected to chemical investigation, contains protein, lipid, carbohydrate, and desoxypentose nucleic acid. The virus preparations also were found to contain phosphatase, catalase, lipase, biotin, riboflavin, flavin-adenine nucleotide, and significant amounts of copper. Observations with the electron microscope of vaccine virus, as well as of certain bacteriophages, have indicated the presence within their particles of an internal structure; possibly a nucleus-like body, a granular appearance, a membrane and in the case of phage a tadpole shape (2, 24). The interpretation is complicated by the irregularity of the dimensions of these massive virus particles. Photomicrographs of the crystals of tobacco mosaic and bushy stunt viruses are shown in Fig. 3. Electron micrographs of a number of representative viruses are reproduced in Fig. 4.

The data now available on viruses indicate that in the transition between the small and large viruses, along with increase in mass there is an increase in complexity of composition, structure, and function. Thus the viruses appear to provide a bridge between proteins and viable organisms. Between bushy stunt virus and vaccine virus, there is found a continuous series of structures of gradually increasing mass and complexity, having in common the biological property of virus activity.

Viruses, like other biologically active proteins, retain their characteristic property of producing disease only when in the intact native state. The viruses are susceptible to digestion by proteolytic enzymes or denaturation by appropriate treatment. Digestion or denaturation of the virus protein is accompanied by loss of virus activity. Tobacco mosaic virus treated with formaldehyde is inactivated and this is associated with a decrease in amino nitrogen and a decrease in the color produced with Folin's phenol reagent. The formalized virus can be partially reactivated and this is accompanied by an increase in amino acid nitrogen and increase in phenol color.

Paralleling the investigations of the essential chemical groups of biologically active proteins, work has been carried on changing the chemical structure of viruses. In tobacco mosaic virus, it is possible to change the chemical structure through the oxidation of sulfhydryl groups, or by introducing several thousand acetyl, phenylureido, carbobenzoxy, benzene sulfonyl, or malonyl groupings. These derivatives are infectious, but the disease which they produce is the unaltered tobacco mosaic disease, and is accompanied by the



FIG. 3. Photomicrographs of crystals of viruses. (A) Tobacco mosaic virus. (B) Bushy stunt virus. Stanley, W. M. (109).

production, not of the respectively altered structures, but of particles of ordinary tobacco mosaic viruses. However, an encouraging result of these chemical studies is that in some instances the chemical variants exhibit a modified virulence for secondary hosts.

Although it is possible to produce considerable alterations in the virus proteins without loss of infectivity, it appears that certain of

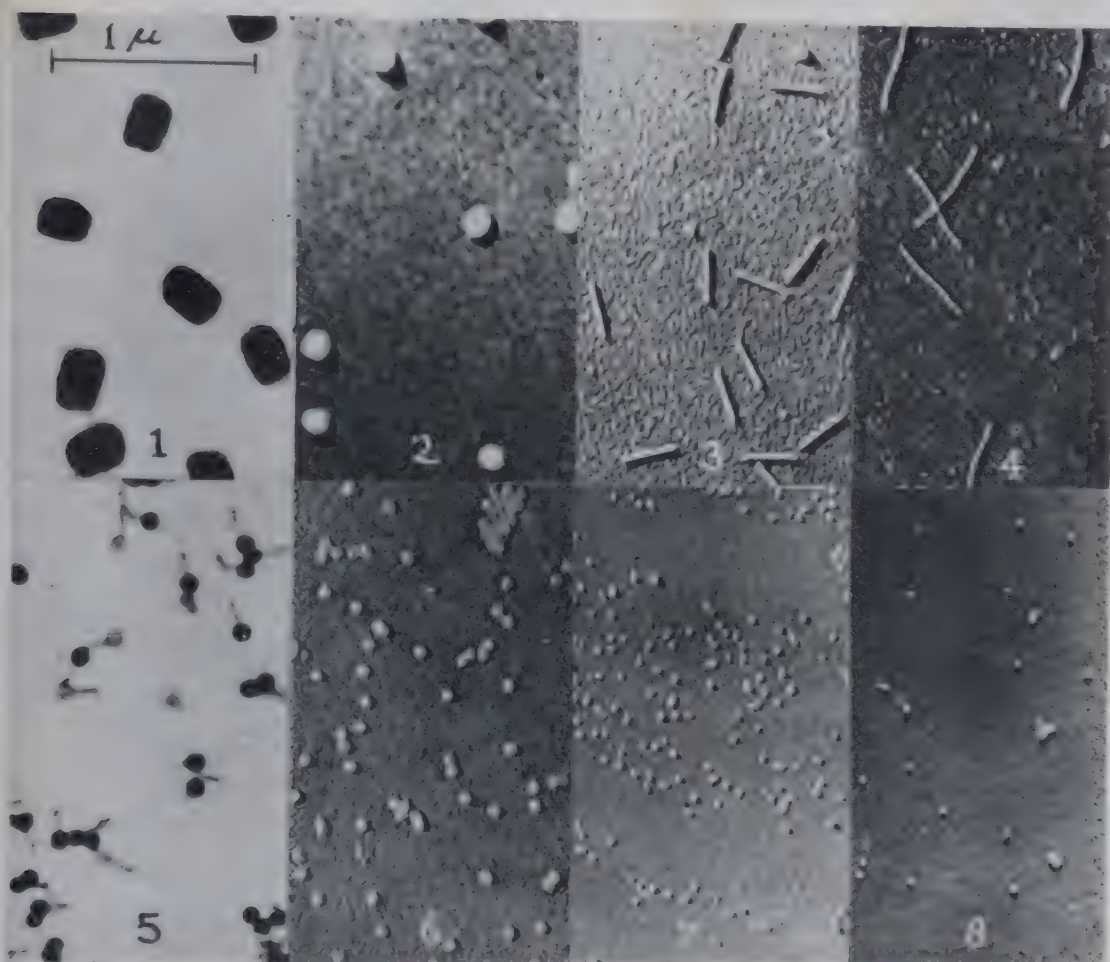


FIG. 4. Electron micrographs of some highly purified viruses. 1. Vaccinia virus. 2. Influenza virus. 3. Tobacco mosaic virus. 4. Potato-X virus (latent mosaic of tobacco). 5. T_2 bacteriophage. 6. Shope papilloma virus. 7. Southern bean mosaic virus. 8. Tomato bushy stunt virus. All mounts except 1 and 5 were shadowed with gold before examination in the electron microscope. Knight, C. A. (64). Courtesy of Dr. W. M. Stanley and Dr. C. A. Knight, Virus Laboratory, University of California.

the amino, phenolic and indole groups of the tobacco mosaic virus are required for its activity. Oxidation of sulfhydryl groups to disulfide linkages resulted in no loss of activity, but only 70% of the amino groups and 20% of the phenol plus indole groups could be substituted and the virus retain complete activity.

The existence of different strains of a given virus, probably pro-

duced by a process analogous to mutation in organisms, has already been mentioned. Tobacco mosaic has proved to be particularly suitable for study of this aspect of viruses. Stanley (109) isolated crystalline nucleoproteins of a number of tobacco mosaic virus strains which possessed similar yet distinctive physical and chemical properties. No differences have been demonstrated in the nature of the nucleic acid among the different strains. On the other hand, striking differences have been found in some instances in the amino acid composition of the protein. Fairly complete amino acid analyses have been carried out on eight strains of tobacco mosaic virus (64). In general, those strains most distantly related to ordinary tobacco mosaic virus differed most from it in protein composition. A striking example is the Holmes rib grass strain which differed in 13 respects, including the possession of histidine and methionine, which are entirely absent from tobacco mosaic virus or other strains. In at least one case, namely, the masked strain, no chemical distinction was found from ordinary tobacco mosaic virus.

An important result of the studies on chemical composition is that it very much dims the hope of producing direct mutations of viruses in the laboratory. As stated by Knight (64): "For example, it is hard to visualize the removal of 1300 lysine residues from, or the incorporation of 2500 glutamic acid residues in, the peptide fabric of a fully formed virus particle by the direct action of chemicals or radiations on the virus particles themselves."

The inference to be drawn from these observations is that the reproduction of viruses is similar to the reproduction of genes in the germ plasm. The mutation of a virus apparently is a discontinuous process resulting in deep seated chemical and structural changes far more profound than those of mere changes in the physical configuration of the virus protein molecule.

The primary pathological changes produced in cells by viruses are either proliferation or degenerative in character. Degenerative changes predominate in such diseases as yellow fever, poliomyelitis, and tobacco necrosis; cellular proliferation is characteristic of Shope rabbit papilloma and tobacco enation mosaic.

Protection may be secured against certain virus disease by immunization. Vaccination against smallpox with attenuated forms of the virus was developed long before the discovery of viruses, or of the development of the sciences of bacteriology and immunology, for that matter. Immunization against yellow fever has been made possible by reducing the virulence of this virus by passage from

man to monkeys, mice and chick embryos. The Pasteur treatment against rabies is another example of immunization against a virus disease. Unfortunately, however, little progress has been made in producing immunity against numerous virus diseases.

2. Bacteriophage

Bacteriophage apparently is a virus produced by certain bacteria which causes the bacterial cells to disintegrate and which multiplies only within the living host cells (26, 54), or at the expense of the bacterial protoplasm (124). Like viruses of higher organisms, a given bacteriophage is specific for one particular strain or closely related strain of bacteria. However, it has been found possible to adapt phage from one strain of bacteria to another. As with other viruses, it has been impossible to demonstrate the existence of any metabolism in bacteriophage.

Assay of bacteriophage is generally carried out by the plaque count method. "A plaque is a virus colony on an agar plate covered with a layer of sensitive bacteria. The bacteria form a continuous sheet of growth on the plate, and a virus colony appears as a circular hole in this sheet, due to the destruction of the bacteria where virus growth has occurred" (26).

Bacteriophage can leave an infected cell only when the cell is lysed. The liberated virus particles then reach other cells by diffusion through the culture medium and become attached to the surface of bacteria which are sensitive to the phage. These cells continue to divide at an undiminished rate for a period, during which the phage rapidly increases. After a certain concentration of the virus is attained the bacterial cells undergo lysis. Usually the time between infection and lysis is around 20 minutes.

Both the bacteria and phage can mutate in the course of culturing. The mutation of the bacteria is evidenced by a change from sensitivity to resistance to lysis. This is a rare occurrence and a culture of a billion bacteria may give rise to a few hundred resistant forms. The phage can also mutate during the multiplication of the virus in its host and this is manifested by an alteration in the host range.

It has been suggested that bacteriophage, and viruses generally, have a structure comparable to a gene complex (chromosome). This would explain the reactivation observed upon irradiating phage with ultraviolet light. The inactive phage may be reactivated by transfer from active phage of the self reproducing units (genes) which have been lost by irradiation. The requirement for reactiva-

tion is that a given genetic locus does not carry a lethal mutation in all of the particles that infect the same cell. From this it may be deduced that growth of phage takes place by the independent reproduction of "unit genetic loci" inside the host cell (80).

The mutant strains of a virus are exemplified by the seven variants designated by the symbols T_1 to T_7 that can multiply in the non-motile "B" strain of *Escherichia coli*. These can be differentiated by serological grouping, cross-resistance grouping, latent period and adsorption rate on the B strain of *E. coli*.

Bacteriophage has been purified by both chemical methods and by differential ultracentrifugation.

Northrop (84) has prepared highly purified bacteriophage from cultures of a non-pathogenic staphylococcus grown on a low protein culture medium prepared from an extract of dried yeast in boiling water. Purification of the phage was difficult because of the presence of a mucin-like material which prevented fractionation by any of the usual protein methods. After removing much of the mucin by lead acetate and filtration through Filter-Cel and subsequently digesting a large part of the inert protein with trypsin, the phage could now be salted out with 0.6 saturated ammonium sulfate.

Several strains of bacteriophage have been purified by centrifugation. Hook and coworkers (57) purified the T_2 strain by preliminary removal of bacterial debris in the Sharples centrifuge, followed by sedimentation of the virus in the same centrifuge and further concentration in the vacuum ultracentrifuge.

The isolated virus material contained protein, lipid, carbohydrate and nucleic acid. Of the total about 50% was protein and 40% nucleic acid; 33.4% being the desoxypentose and 6.6 the ribopentose type. Phage is digested by chymotrypsin but is resistant to trypsin or pepsin. The pH range of stability with respect to infectivity extended from pH 5 to 9.

Photographs of phage with the electron microscope (2, 26, 57) have revealed characteristic tadpole-shaped particles with a large head consisting of particles enclosed in a well-defined membrane and a flagellar appendage. The dimensions of the head piece are about $100 \times 80 \text{ m}\mu$ and of the tail piece $110 \times 18 \text{ m}\mu$; the over-all length of the virus being about $210 \text{ m}\mu$ (57).

Wyckoff (124) has made observations with the electron microscope which lend themselves to the interpretation that bacteriophage particles increase in number at the expense of the protoplasm of lysed bacteria, in a manner that resembles the multiplication of

coccoid bacteria. Wyckoff points out that bacteriophage resembles living micro-organism in their complex organization, in their abnormally rapid rate of diffusion, which may represent independent movement by means of the flagella, and the fact that the amino acid composition of bacteriophage protein is distinctly different from that of its host.

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Chapter XIII

THE METABOLISM OF AMINO ACIDS AND PROTEINS

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I. INTRODUCTION

THE METABOLISM of protein will be considered in three parts, first from the point of view of the reactions of the amino acids and the fate of the products, that is, from the point of view of intermediary metabolism; then, the second part will deal with the general picture of protein metabolism; and lastly, the metabolism of individual amino acids will be considered. Because more is known concerning protein metabolism in mammals than in other organisms the treatment of the subject will be oriented toward these species in order to derive as clear a picture as possible of the way in which protein is handled.

The metabolism of protein necessarily presents a more complex problem than that of either fat or carbohydrate, for, quite obviously, protein metabolism involves both a study of the metabolism of nitrogen as well as of carbon chains and rings. It is further complicated because this substance is not only the most significant protoplasmic component but is also an essential food. So, under some circumstances, energy requirements can be met by the catabolism of protein, the protein broken down being either of endogenous or of exogenous origin. When catabolism of endogenous protein takes place there follows a net reduction in the metabolic propensities of the tissues and of the organism as a whole, not only with respect to protein but also with respect to all other metabolites. The protein component of the tissue is enzymic in nature so any change in size of this component will be reflected in quantitative changes in the metabolism as a whole. In contrast, a major part of the fat and carbohydrate component of the organism represents stored material only, so that its reduction or increase will not interfere so vitally with the metabolism of the remaining protoplasm.

The general picture at the present lacks clarity largely due to the impact of studies with labeling agents, most particularly with N^{15} and C^{14} , on a classical picture of metabolism which largely resulted from the studies and interpretations of Folin. Folin placed a clear line of demarcation between two forms of metabolism—

endogenous and exogenous—but such a point of view has become untenable in view of the recent experimental work with isotopes. Yet the concept of endogenous and exogenous metabolism is not without value.

Formerly, the production of creatinine was taken to indicate the extent of the endogenous metabolism. This has now been shown to be incorrect. Also the implication that tissue protein is a static entity which undergoes little change except that due to “wear and tear” is shown to be untrue. In view of the classical histological and cytological knowledge and the long known regenerating power of tissues such as liver, it is indeed strange that such a static point of view should have been seriously entertained. It is also surprising in view of the known enzymic properties of tissues which are such as to cause a rapid autolysis as soon as there is a failure of the energy supply. A static system implies a complete inactivation or localization of such enzymes until such times as exogenous substrate is present—a well nigh impossible situation to conceive.

The breakdown of amino acids in the organism proceeds so that in general there is an early separation of the nitrogenous part of the molecule from the carbon chain. Thereafter, the two parts follow quite different pathways in metabolism. Consequently, if the removal of the amino group is blocked by acylation, catabolism of the amino acid may be stopped. In mammals, the nitrogen once removed, is converted in major part into urea, which appears in the urine; in birds it appears as uric acid in the excreta. Lesser amounts of catabolic nitrogen are eliminated in other forms such as ammonia, creatine-creatinine, and uric acid. In some species allantoin replaces uric acid. Many of the aliphatic nitrogen-free compounds which are formed by the breakdown of the amino acids are identical with products which arise during the metabolism of carbohydrate, *e.g.*, pyruvic acid (alanine), oxalacetic acid (aspartic acid), or from the breakdown of fat, *e.g.*, acetate or acetoacetate (leucine, tyrosine). Consequently, a large part of protein carbon and hydrogen follows the same pathways in metabolism as that of carbohydrate and fat carbon and hydrogen.

As in the study of the mechanism of fat breakdown, nearly all the early knowledge was derived by characterization of the breakdown products of aromatic substituted amino acids in normal animals. Studies conducted on individuals with the metabolic derangement, alcaptonuria, provided further evidence. The benzene ring was shown to offer so much greater resistance to attack

by the catabolic system that it was possible to isolate a variety of intermediary products. Thus, from amino acids there were isolated compounds with the same number of carbon atoms, *e.g.* acid I, keto acid II, hydroxy acid III, unsaturated acid IV, and the acetyl derivative of the original amino acid V, as well as various compounds with one less carbon atom (VI, VII, VIII).

- | | |
|-------------------------|----------------------------|
| I. $R-CH_2-CH_2-COOH$ | V. $R-CH_2CHNHCOCH_3-COOH$ |
| II. $R-CH_2-CO-COOH$ | VI. $R-CH_2-CH_2-NH_2$ |
| III. $R-CH_2-CHOH-COOH$ | VII. $R-CH_2-CH_2OH$ |
| IV. $R-CH=CH-COOH$ | VIII. $R-CH_2-COOH$ |

Products I, VI, and VII are prominent in the breakdown of amino acids in bacteria but are of less importance in animals except for certain amines which possess physiological activity. These amines, however, can hardly be on the main catabolic pathway in animals because of their great physiological activity and because some are not readily broken down in animal tissues. Therefore, it is not probable that acid VIII will arise by the oxidation of VI or VII but rather by the decarboxylation and oxidation of previously deaminated residues. Hence, the first problem to elucidate is the process which leads to products like II and III and the significance of the unsaturated acid and acetyl derivatives.

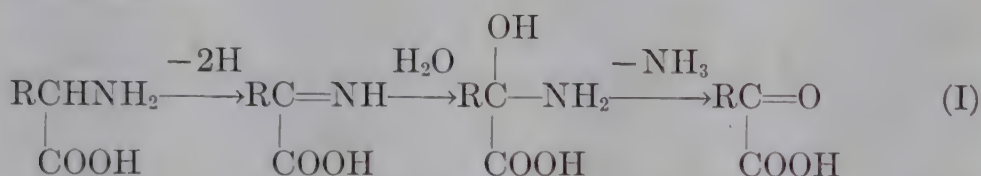
II. DEAMINATION AND REAMINATION OF AMINO ACIDS

1. General Considerations (373a)

This problem was first attacked by Neubauer (459) who fed phenylglycine to several species of animals. From the urine he succeeded in isolating mandelic acid ($C_6H_5CHOHCOOH$) and phenylglyoxylic acid ($C_6H_5COCOOH$). When this keto acid was fed, mandelic acid again appeared in the urine, but after feeding the alcohol, only traces of the keto acid were formed. From these results, it appeared more probable that the primary deamination product was the keto acid with the alcohol arising by a secondary reduction. Later, the same reactions were shown to proceed in the perfused liver and in breis of the same tissue as well as in yeast (462). Additional evidence was provided by the behavior of *p*-hydroxyphenyl lactic acid and the corresponding keto acid in the alcaptonuric (461) and in rabbits (360, 368). Human subjects with this metabolic anomaly form homogentisic acid from tyrosine or *p*-hydroxyphenyl pyruvic acid but not from the substituted

lactic acid (459). Hence, the metabolic path is presumably through the keto acid. These results and conclusions were confirmed by others using various ring substituted phenylglycines (252) and *p*-chlorophenylalanine (249).

Knoop (342, 346) found that the deamination could be reversed. On feeding γ -phenyl- α -aminobutyric acid to a dog, the acetyl derivative appeared in the urine as well as γ -phenyl- α -hydroxybutyric acid (see also 465). When γ -phenyl- α -ketobutyric acid was injected, the same products were found with the hydroxy acid in greater yield than before. In both cases benzoic acid conjugated with glycine (hippuric acid) was also present in the urine. Knoop considered these results to be in agreement with those of Neubauer, and believed the hydroxy acid to be a secondary product. He proposed the following mechanism:



The postulated intermediates in the formation of the keto acid were anticipated to be very unstable. The acetyl derivative was presumed to arise by a reaction in which the keto acid, pyruvic acid, and ammonia participated to give acetyl amino acid and carbon dioxide (see under section 4); and the benzoic acid by decarboxylation of the keto acid followed by oxidation according to the β -oxidation mechanism prevailing in the fatty acid metabolism.

2. D-amino Acid Oxidase

Later work has resulted in the discovery of systems which support the conclusions of Knoop and Neubauer. Tissue slice and enzyme preparations have been shown to be capable of deaminating amino acids in the presence of oxygen with the production of keto acids and ammonia. Among the first experiments of this type were those of Meyerhof, Lohmann, and Meier (437), who showed that liver slices could deaminate alanine with the production of NH_3 and with an increase in oxygen consumption. Ammonia production decreased under anaerobic conditions or in the presence of cyanide. Kisch (336) later showed that kidney tissue exhibited a greater rise in oxygen consumption in the presence of amino acids than did liver under similar conditions.

However, it remained for Krebs (370) to make a thorough investigation, using both tissue slices and crude extracts. He examined

the influence of a wide variety of amino acids on the ammonia production, the keto acid formation, and the oxygen consumption of slices of liver, kidney, and many other tissues, particularly from rats. The extra oxygen consumed in the presence of amino acid was, in many cases, in the ratio of one mole utilized per two moles of ammonia formed. Where this relationship did not hold, it was shown that the keto acid formed was being oxidized. This oxidation could be prevented by adding arsenite and did not proceed at all in extracts. No cases were found in which less oxygen was consumed than that required by the equation:



Thus, hydrolytic deamination apparently did not proceed. Keto acids, such as phenylpyruvic acid from the oxidation of phenylalanine, did accumulate due to their resistance to further oxidation. Other keto acids accumulate in preparations of intestinal mucosa (456). The keto acids may be determined directly as the 2,4-dinitrophenyl hydrazones (535) or alternatively they can be fixed as bisulfite compounds (60) or determined by oxidation with ceric sulfate (372).

In the liver all the ammonia is immediately converted to urea.

Either oxygen or methylene blue (55) may serve as hydrogen acceptor in extracts but the reaction proceeds more slowly with the dye. Cyanide inhibits the deamination in slices but not in extracts.

The most surprising thing established by the experiments of Krebs, and confirmed by all other workers, is that, with the exception of the dicarboxylic acids, the amino acids of the D configuration are deaminated on the average ten times as fast as those of the corresponding L configuration (Table I).

TABLE I
RATE OF DEAMINATION OF L- AND D-AMINO ACIDS
IN RAT KIDNEY SLICES

	L	D
Alanine	2.03	36.5
Valine	2.53	56.5
Leucine	5.35	33.6
Phenylalanine	9.07	75.7
Histidine	1.85	8.42
Aspartic acid	13.9	1.26
Glutamic acid	7.73	—

Data expressed as Q_{NH_3} . Amino acid concentration = 0.05 M. Q_{NH_3} = cubic mm. of ammonia produced per mg. of dry tissue per hour.
Krebs, H. A., (370).

Due to the observed difference in behavior between the D and L-isomers in slices and also in extracts and because the deamination of only L-isomers could be inhibited in slices by octyl alcohol, Krebs came to the conclusion that there were two different deaminating systems. One was separable from the cell, the other was inseparable. He suggested that the D-system might be a fraction of the L-system.

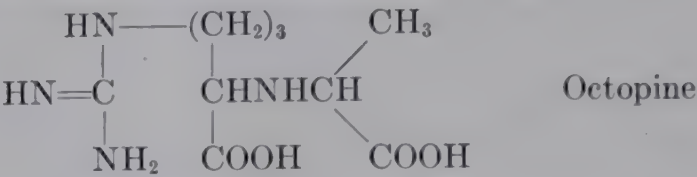
Many other workers helped to define the system responsible for the deamination of the D-amino acids more fully and confirmed all the main findings of Krebs (60, 334). The kidney, and particularly the cortex, appears to be most active in deamination (334, 389). In some species the liver is only one tenth as active as the kidney but in others there are not such wide differences (335, 456). Oxidative deamination in brain has been observed (205) and the activity of intestinal mucosa is also not inappreciable (456). In the intact animal, the liver is probably of primary importance because of its strategic location, relatively large size, and ability to take up amino acid.

The relative rates at which different D-amino acids are attacked vary considerably in different species (335). Extracts of beef and horse kidney attack β -alanine with avidity, although the kidneys from most other species do not attack this amino acid. Some kidneys attack glycine readily (horse, ox, guinea pig), whereas in most kidneys this amino acid is resistant. This suggests that different enzymes are concerned in the deamination, and these have been demonstrated in the cases of glycine and serine. It should not be forgotten that the specificity of the oxidase may depend to some extent on the source of the protein part of the enzyme (328). Also, the absolute rates at which any one amino acid is attacked vary very considerably with the species. Generally, there is a larger amount of deaminating enzyme in the livers of carnivorous animals than in those of herbivorous (69).

The D-amino acid oxidase is capable of deaminating the N-monomethyl amino acids, *e.g.*, N-methylalanine, but not the N-dimethyl amino acids, which, according to the classical formulation, have no hydrogen on the nitrogen (331, 286). Amino acids with other substituents on the nitrogen (N-ethylalanine) are more slowly deaminated than the parent compounds or are not deaminated at all (N-acetyl and N-butylalanine) (326). The deamination of a series of N-monomethyl amino acids was examined by Handler, Bernheim, and Klein (286). The derivatives of methionine and leucine were readily deaminated, but those of valine, his-

tidine, phenylalanine, and tryptophan were not attacked. These results are not in complete accord with the results of feeding the same derivatives to animals deficient in the corresponding essential amino acids. For instance the N-methyl derivative of histidine supports growth, so presumably it is deaminated and the keto acid reaminated.

α,α' -Iminodicarboxylic acids such as octopine are not oxidatively



deaminated (325, 326). The presence of hydroxyl, sulfhydryl, or of extra amino groups inhibits the deamination of the aliphatic amino acids (510) and the hydroxyl and sulfhydryl amino acids are deaminated by a special enzyme system (150).

Klein and Handler (339) and others (230, 327) have compared

TABLE II
OXIDATION OF D- AND L-AMINO ACIDS

	D-amino Acids				L-amino Acids
	% Deaminated (1)	% Oxidized (2)	Relative Oxidation Rate (3)	Relative Oxidation Rate (4)	Relative Oxidation Rate (5)
Glycine	0	0	—	0	—
Alanine	107	94	224	39	8
Leucine	35	87	100	100	100
Isoleucine	—	94	102	57	71
Valine	100	94	—	39	28
Serine	11	18	49*	0	—
Threonine	—	18	—	0	—
Cystine	—	0	—	—	>15
Methionine	—	90	294	150	81
Aspartic Acid	99	24	47*	44	—
Glutamic Acid	11	0	—	64	—
Proline	—	89	290	0	77
Phenylglycine	—	20	—	14	—
Phenylalanine	99	87	84	128	45
Tyrosine	100	60	—	—	>20
Tryptophan	—	22	—	8	40
Arginine	15	19	45*	120	—
Lysine	0	0	—	21	—
Histidine	21	16	49*	—	9

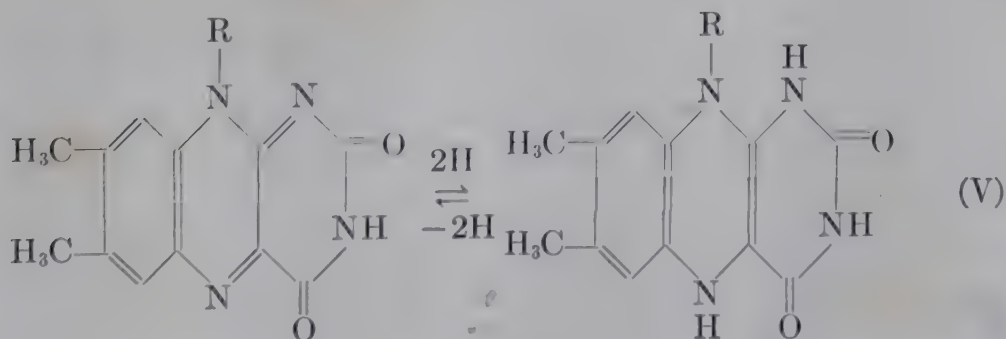
(1) Felix, K., and Zorn, K. (230)—percent deamination as measured by ammonia production. Enzyme from acetone dried powder of pig kidney.
(2) Klein, J. R., and Handler, P. (339)—percent of amino acid oxidized, using reconstituted enzyme system.
(3) Karrer, P., and Frank, H. (327)—relative oxidation rate (leucine = 100), using reconstituted enzyme system. Results with asterisks evidently not considered significant by the authors.
(4) Horowitz, N. H. (312)—relative oxidation rate (leucine = 100), using oxidase from *Neurospora crassa*.
(5) Blanchard, M., Green, D. E., Nocito, V., and Ratner, S. (70)—relative oxidation rate (leucine = 100), using purified L-amino acid oxidase.

the deamination of a large number of amino acids by more or less purified enzymes. It has been found by most workers that lysine is not deaminated (229, 468), although this is disputed by one observer (197). It is seen from Table II that serine, glutamic acid, arginine, and histidine (228) are only slowly attacked. β -alanine is not attacked at all in most species and in the intact animal it has been observed that other β -amino acids are not metabolized but are excreted unchanged (380). Aminoisobutyric acid, and α -methyl- α -aminoisovaleric acid are resistant to attack but dopa (3,4-dihydroxyphenylalanine) is quite readily deaminated as are also aminobutyric acid, norvaline, and norleucine (271, 70).

A closer insight into the mechanism of the action of the oxidase is provided by the work of Keilin and Hartree (331). These authors found that hydrogen peroxide was one of the products. In the presence of peroxidase the peroxide produced could be used to bring about secondary oxidations such as the oxidation of alcohol to aldehyde (331) or hemoglobin to methemoglobin (60).



Unless specially purified, D-amino acid oxidase preparations contain sufficient catalase to decompose the hydrogen peroxide to water and oxygen so that the net utilization of oxygen is one atom per mole of amino acid deaminated. The enzyme, therefore, acts like other flavoproteins such as xanthine oxidase. The nature of the enzyme was established by Warburg and Christian (637) and by Straub (592, 171). It consists of a specific protein combined with a nucleotide containing isoalloxazine and adenine. The isoalloxazine nucleus of the complete enzyme is capable of being reversibly hydrogenated and dehydrogenated in the presence of an appropriate amino acid as hydrogen donor and when oxygen or a suitable dye is present, as hydrogen acceptor.



The protein has been prepared in a pure state by Negelein and Brömel (458). Stadie and Zapp (577) have studied the combination between protein, prosthetic group and substrate. On oxidation of the sulfhydryl groups of the protein the enzyme becomes inactive (561).

Deamination of D-amino acids in brain, liver, and kidney, and even in reconstructed enzyme systems, is inhibited by relatively high concentrations of L-amino acid (197). The oxidase is also inhibited more or less specifically by benzoic acid (341) and its derivatives (33). The purified enzyme is reported to be activated by traces of either D- or L-amino acid, in particular by histidine or proteins containing large amounts of this amino acid (206).

Horowitz (312) has found that *Neurospora crassa* is capable of deaminating D-amino acids. In most respects the enzyme is similar to that extracted from mammalian kidneys. It is inhibited by isovaline in a competitive way but is not affected by benzoic acid. The presence of this D-amino acid oxidase quite evidently partly explains the ability of mutants of the mold, which require specific amino acids, to grow when the appropriate D-amino acid is supplied.¹

3. L-amino Acid Oxidase

In his pioneer investigations, Krebs showed that with the exception of the dicarboxylic acids the L-amino acids are not deaminated at an appreciable rate either in tissue slices or by the readily extracted enzyme. The behavior of these dicarboxylic amino acids has been investigated in detail by Kögl and coworkers (348). Edlbacher and Grauer (198) have examined the effect of inhibitors on the deamination of the natural isomers of alanine phenylalanine, aspartic acid, and glutamic acid in kidney slices. From their results summarized in Table III it can be seen that the systems responsible for these four deaminations are different. Additive effects on ammonia production are produced by the simultaneous deamination of alanine, phenylalanine, and aspartic acid. Green and coworkers (271, 70) have prepared a pure enzyme from rat kidney and liver which is capable of oxidatively deaminating L-amino acids. However, the enzyme has little or no effect on glycine, serine, threonine, the dicarboxylic acids, lysine, arginine or

¹ Technically, the D-amino oxidase has been used to prepare L-amino acids from the DL-mixture by removing the D-amino acid as the product of deamination. For example, Duschinsky and Jeannerat and others (191, 43) obtained a yield of 83.5 percent of L-alanine from 9 gm of a DL-mixture. Several other L-amino acids have been prepared by similar methods.

on D-amino acids. The enzyme is different from the D-enzyme in respect to its prosthetic group which is not flavin adenine dinucleotide, but is simply riboflavin phosphate. Its activity is of a low order compared to that of other known flavoproteins. It is not inhibited by benzoic acid but may be inhibited somewhat by D-amino acids. With respect to the relative velocity with which various amino acids are deaminated, reference to Table II will show that the L-oxidizing enzyme behaves similarly to the D-amino acid oxidase. The mechanism of the reaction for both enzymes is the same.

TABLE III
EFFECT OF INHIBITORS ON DEAMINATION OF L-AMINO
ACIDS IN KIDNEY SLICES

Inhibitor	Molarity	Alanine	Valine	Phenyl- alanine	Aspartic acid	Glutamic acid
Cyanide	0.001	+	+	+	+	+
Iodoacetate	0.001	+	+	+		-
Arsenite	0.001	+	+	-	-	-
Malonate	0.05	+	+	+	+	-
Pyrophosphate	0.02	-	-	(+)		-
Fluoride	0.1	+	+	+		-

Edlbacher, S., and Grauer, H. (198).

Another enzyme acting on the L-form of amino acids is found in snake venoms (669). In bacteria (*Proteus vulgaris* and many other species) there are at least two enzyme systems responsible for the deamination of amino acids (593). All amino acids tested are broken down in fresh bacteria but as the preparations age, they lose the capacity to break down glycine, alanine, valine, serine, threonine, aspartic and glutamic acids, proline, and lysine. The more stable type of oxidase acts on the L-amino acids to produce keto acids and ammonia but no hydrogen peroxide is formed. It even acts on arginine in this fashion. The enzyme appears to be associated with the small particles in the bacterial cell. The optimum reaction is at pH 7 and it is not inhibited with benzoate. Thus the bacterial enzyme differs in three important respects from the animal enzyme. The general problem of amino acid breakdown in bacteria has been dealt with by Gale (255).

Thus, L-amino acid oxidase of animal tissues has, in part at least, the properties of a system which must be postulated to account for the breakdown of L-amino acids, but it cannot supply a complete answer to the problem for the following reasons:

1. Little of the enzyme can be extracted from the kidneys of animals other than the rat.
2. The catalytic activity of the enzyme is very weak.
3. The enzyme does not attack many amino acids at an appreciable rate.

Therefore, it is necessary to assume either (a) that the extraction or detection methods are at fault, or (b) that there are specific enzymes responsible for the deamination of some of the amino acids, or (c) that deamination follows attack on some other part of the molecule, or (d) that deamination is coupled with some other reaction (see sections 5, 6, 7). The third situation appears to prevail with respect to histidine and lysine. Specific deaminating enzymes exist and these will be dealt with individually.

This leaves unsettled the status and function of the D-amino acid oxidase. Krebs suggested that this enzyme is a fraction of the whole enzyme responsible for the breakdown of L-amino acid, but chemical work does not support this suggestion. The actual existence of the enzyme in active form in the animal is apparently demonstrated by the results of feeding D-amino acids which in many cases are rapidly deaminated with the resultant appearance of keto acids in the urine (635), and in some cases by the conversion of the D-isomer to the L-isomer (see Chapter X). Other workers have proposed that the enzyme may serve to remove D-amino acid formed by racemization of the natural isomers or by *de novo* synthesis within or without the organism. However, it is very improbable that D-amino acids are formed in the ordinary course of metabolism (see section 9). Again, the D-amino acid oxidase may arise due to inversion of optical specificity when the enzyme is extracted. In experiments with tissue slices, it is found that a considerable fraction of the enzyme rapidly diffuses out of the cells into the medium but just how much of the breakdown is due to this extracellular component is not known.

A surprising property of the L-amino acid oxidase not possessed by the D-enzyme is the ability to oxidize hydroxy acids to keto acids (71).



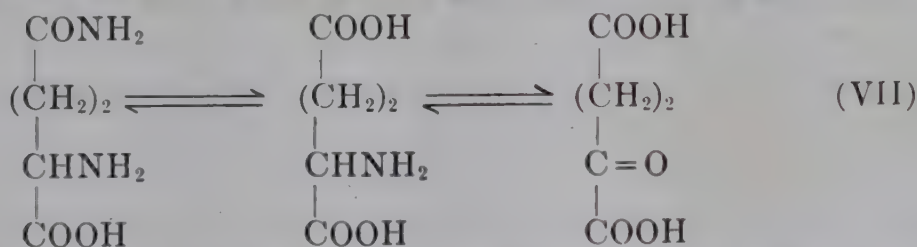
In fact, the hydroxy acids are oxidized several times as fast as the corresponding amino acids. The enzyme is distinct from lactic and malic acid dehydrogenases. Thus, it appears possible that the deaminating property of this amino acid oxidase is an incidental

one and that the deamination of L-amino acids may proceed by some entirely different mechanism.²

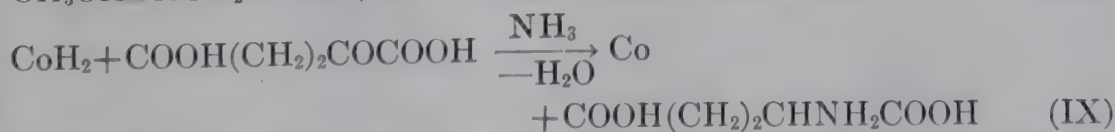
4. Deamination and Reamination of Specific Amino Acids

a. Glutamic acid, glutamine and aspartic acids

Thunberg in 1920 (611) showed glutamic acid to be the only amino acid oxidized at a significant rate in muscle breis in the presence of methylene blue as hydrogen acceptor. This was confirmed by other workers who discovered that the enzyme system is widely distributed in the plant and in the animal kingdoms (222, 10); so it appears that glutamic acid occupies a special position with respect to its oxidative deamination. In brain, Weil-Malherbe (641) found that this amino acid was the only one which maintained the respiration out of a large number tested. D-glutamic acid had no similar effect. The products of the reaction were α -ketoglutarate and glutamine; thus, the amino acid was partly deaminated and partly converted to the amide. In the presence of oxidizable substrate, the reaction was reversed so that α -ketoglutarate could be converted back to glutamic acid and glutamine.



The formation of glutamine from glutamic acid and ammonia had been previously investigated by Krebs (371, 475). Weil-Malherbe claimed that there was a reversal in the stereochemical specificity of the enzyme—a result of its extraction from the tissue. Euler and coworkers (221, 9) and Dewan (188) have shown that the enzyme requires either di- or triphosphopyridine nucleotide (Coenzyme I or II) in animals, but in yeast Coenzyme II is required specifically. In the presence of systems which can reduce the coenzyme, glutamic acid or glutamine is synthesized. Such a system is provided by β -hydroxybutyric acid, its specific dehydrogenase and the coenzyme. The reactions may be written:



² The L-amino acid oxidase of bacteria has been used to prepare D-amino acids (593).

In some tissues, such as heart, muscle and kidney, but not in liver and brain, amination can occur at the expense of energy derived from the anaerobic decarboxylation of the α -ketoglutarate (374):



In addition to the two types of coupling mentioned, the amination may be articulated with the oxidation of isocitrate to α -ketoglutarate and carbon dioxide (9) and by that of malate to oxaloacetate as shown by Krebs and coworkers in a recent study (374b). One surprising thing about this amination reaction is that it proceeds more rapidly—three to 15 times—in homogenates of liver than in liver slices. In the kidney cortex, on the other hand, no such contrast exists.

The reaction under consideration is highly reversible so that depending on the conditions glutamic acid is either anabolized or catabolized. The deamination presumably proceeds by dehydrogenation to give the imino acid (220). There is no reduction of α -ketoglutarate to α -hydroxyglutarate in the presence of this specific dehydrogenase, nor is the dehydrogenase capable of bringing about the amination of other keto acids such as oxalacetic acid, α -ketobutyric acid, or pyruvate. Likewise, amination with methylamine does not proceed. Thus, the enzyme shows a high degree of specificity and differs from the amino acid oxidases so far described in that it requires a coenzyme. α -Ketoglutaric acid is the only keto acid which has been convincingly demonstrated to give rise to the corresponding amino acid *in vitro*.

Euler and coworkers were able to extract twice as much of the enzyme from liver as from kidney, with extracts of other tissues containing only one tenth as much as liver.

According to Krebs (371) glutamine synthesis from glutamic acid proceeds in slices of kidney, brain and in the retina of the several species tested. The synthetic system was poisoned by arsenite, and this addition also led to the deamination of the added glutamate. This is presumably due to the failure of the energy supply resulting from the inhibition of α -ketoglutarate oxidation. However, in retina the reactions proceed anaerobically, hence the energy supply here must come from the glycolytic reactions. Pigeon liver slices were shown to be very well suited to the investigation of glutamine synthesis (475) and Speck (572) succeeded in obtaining synthesis in homogenates and even in extracts of the acetone dried powders from this tissue. The enzyme has been shown to be present in the livers,

kidneys and brains of all species tested, and Elliot has particularly investigated the last named tissue (211b). The requirements for the synthesis in pigeon liver homogenates were shown to be: glutamate, ammonia, phosphate, Mg ions and oxygen (572). Generally adenosine triphosphate or muscle adenylic acid addition is necessary to maintain a maximum rate of synthesis because of inadequate conservation of these substances in the homogenate. However, as with other similar systems, when the concentration of adenosine triphosphate is made too high, inhibition rather than stimulation results. Fluoride appears to have a specific inhibiting effect. When instead of homogenates acetone dried powders of pigeon liver are employed Speck was able to show that glutamine synthesis proceeded according to the following reaction (no oxygen being required):



No reaction between glutamic acid and NH_3 occurred in the absence of adenosine triphosphate. It is very interesting to note that this reaction is not specific for ammonia but that this base may be replaced by hydroxylamine, methyl amine or hydrazine. With the first named the product is a hydroxamic acid which may be determined colorimetrically.



In contrast to the relative non-specificity of the reaction with respect to base, with respect to acid the specificity appears to be complete, so that none of a large number tested was able to replace the glutamic acid. However, it has been shown by Elliott and Gale (211c) with a bacterial enzyme and by Speck with the liver enzyme, that there is inhibition of synthesis by methionine sulfoxide, with the bacterial enzyme showing a much greater sensitivity. This inhibition is competitive with respect to glutamate. In view of the evidence mentioned, in particular, of the relative non-specificity of the base, it may be assumed tentatively that the synthesis proceeds in two steps, the second of which requires little activation and may proceed spontaneously:



In plants the synthesis of asparagine may perhaps proceed in the same general fashion. Due to the formal resemblance between the synthesis of glutamine and the synthesis of peptide bonds of various

types, the properties of these systems should be compared (section V, 5, 6).

It has recently been reported that D-aspartic acid is deaminated by a specific enzyme which occurs in rabbit kidney and liver (588a).

b. Glycine and sarcosine

Glycine is subject to deamination by a specific enzyme discovered by Ratner and coworkers (495). The enzyme is present in the liver and kidney of all species examined, with the exception of rat kidney. The enzyme acts specifically on glycine and sarcosine, being without action on N-dimethylglycine, other substituted glycines and peptides. The product of the reaction, glyoxalic acid was isolated as the 2,4-dinitrophenylhydrazone.



Methylene blue can replace oxygen. The prosthetic group of the enzyme is flavinadenine dinucleotide but, per mole of prosthetic group, the enzyme only shows one fourteenth the activity of D-amino acid oxidase. It is questionable whether the operation of this enzyme system can account for the observed rate of glycine catabolism in the whole organism. Glycine may be metabolized following its conversion to serine (see section X, 1).

An enzyme described by Handler and others (286) attacks sarcosine with the production of formaldehyde.



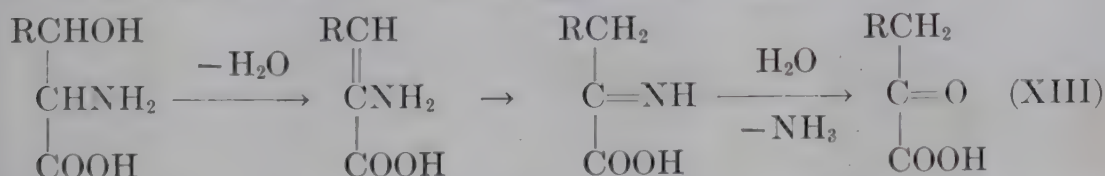
Other L-N-methylamino acids may be similarly demethylated to give formaldehyde according to the experiments of Ling and Tung (405). As previously noted, the D-N-methylamino acids are broken down by the D-amino acid oxidase to produce methylamine provided both hydrogen atoms in the amino group are not substituted. Thus, the D- and L-isomers of this type of amino acid derivative appear to be metabolized via different pathways. Throughout the text other instances will be noted where the D-amino acids themselves are metabolized in different fashion from the corresponding L-isomers.

It appears that the deamination reaction for glycine cannot be reversed, at least to any considerable extent as judged from the data of Shemin (545), who diverted the pathway of glycine metabolism by injecting benzoic acid into rats and guinea pigs. This resulted in the formation of hippuric acid (section V, 1). If labeled

glycine is fed along with the benzoic acid then labeled hippuric acid is found in the urine, with the label appearing in less concentrated form (Table VIII). Consequently, part of the glycine used in the synthesis of the hippuric acid comes from that injected and the rest is of endogenous origin (since the animals were fasted). When Shemin labeled the glycine in both the carboxyl group (C^{13}) and the amino group (N^{15}) the ratio in which the labels originally existed in the molecule was maintained in the glycine residue of the hippuric acid. Hence no glycine could have been formed by amination of labeled C_2 residues resulting from previous deamination of the labeled glycine, otherwise there would have been dilution of the N label relative to the C label. Likewise an efficient direct transfer of nitrogen from glycine to other compounds appears to be eliminated. However, it is known that sarcosine is able to provide glycine for the purpose of conjugation (2), whereas N-ethylglycine cannot function in this manner. N^{15} -labeled sarcosine also readily labels the glycine of proteins and is utilized almost as well as glycine for creatine formation (88).

c. Serine and threonine

Both serine and threonine undergo an anaerobic type of deamination which is not possible in amino acids lacking an alcoholic group in the β position (150). This deamination takes place in bacteria (262) and in liver tissue. The suggested mechanism is:



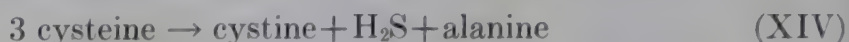
In conformity with this scheme O-alkylated serines are not deaminated. Thus, the enzyme can be considered as a dehydrase similar to the one converting 2-phosphoglyceric acid into 2-phosphoenolpyruvic acid, and to the desulphydrase of cysteine (64). The liver enzyme acts on both D- and L-serine. It is activated by Mg^{++} .

In kidney there is a different enzyme which converts D-serine into β -hydroxypyruvic acid (573). According to Stöhr (590) threonine is converted by oxidation and oxidative deamination into α,β -diketobutyric acid.

d. Cysteine and homocysteine (250, 565)

Animal tissues contain an enzyme which is capable of removing

sulfur from cysteine. This was thought to result in the formation of hydrogen sulfide and alanine.



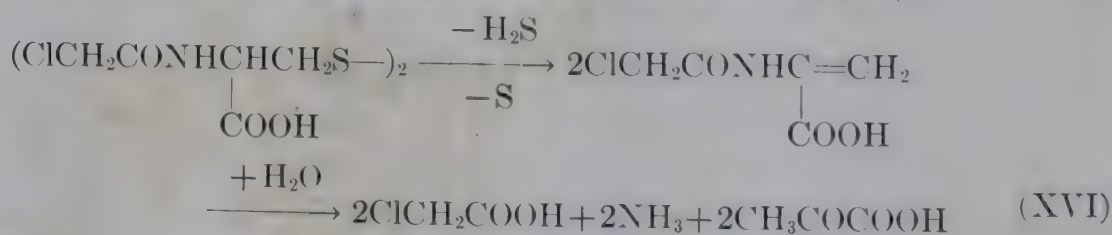
Smythe, however, believes that there is a removal of both ammonia and H_2S by the enzyme (564).



The reaction is analogous to that taking place with serine (see equation XIII) (64). Part of the overall reaction appears to possess a low degree of reversibility as shown by studies with radioactive hydrogen sulfide in the presence of cysteine and the enzyme (566). Traces of radioactive cysteine were produced, but no reversal of the reaction from pyruvic acid was observed. This is in agreement with the essential nature of cysteine-cystine in the diet in the absence of excess methionine: sulfide sulfur is not utilized by mammals to a significant extent.

The enzyme (cysteine desulfhydrase) has been partly purified (385, 386, 421). It is inhibited by cyanide, and carbonyl reagents and activated by Mg^{++} . Whether the same enzyme or another acts on homocysteine is not clear, but the enzyme is less active with this substrate (250).

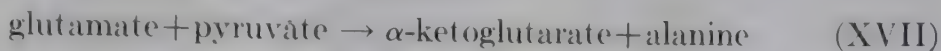
It is also possible for cystine peptides to break down to give hydrogen sulfide, sulfur and dehydropeptides which are, in turn, decomposed to pyruvic acid and ammonia (274, 276).



5. Transamination (298, 155, 125)

In 1930 Needham reported the disappearance of glutamic acid from muscle breis with the appearance of succinic acid, but without being able to account for the nitrogen either in the form of free ammonia or amide nitrogen (457). She concluded that some other substance must have been aminated, but it was not until 1937 that Braunstein and Kritzman (128) discovered the nature of the reaction which occurred. These authors showed that muscle tissue preparations catalyzed the deamination of L-glutamic acid without the formation of free ammonia, but with the simultaneous conversion of pyruvic acid to alanine. This alanine was also of the L configuration (124). The reaction, which proved to be reversible,

was formulated as one of transamination, and the enzyme involved was later referred to as aminopherase.



In contrast to oxidative deamination, the reaction was found to proceed anaerobically in the presence of pyruvic acid. Aerobically, either pyruvic acid or lactic acid would serve because under these conditions, the lactic acid was oxidized to pyruvic acid. *There was never any formation of free ammonia except such as could be attributed to side reactions.*

Braunstein (124) claimed that, with the exception of glycine, the same type of reaction would take place between any α -amino acid (even α -aminobutyric and α -aminocaproic) and the two keto dicarboxylic acids, α -ketoglutaric and oxalacetic. The only restriction found was that one of the reacting acids had to be dicarboxylic. These claims were not supported by other workers and have been deemphasized by Braunstein as a result of later work. Using pigeon breast muscle as enzyme source and α -ketoglutarate as acceptor, Cohen (153) found that the most active amino acids were aspartic, alanine, α -aminobutyric and valine, in the order named. Other amino acids proved to be relatively inactive (Table IV).

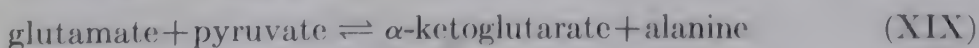
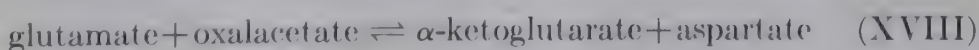
TABLE IV
GLUTAMIC ACID FORMED FROM α -KETOGLUTARATE AND
AMINO ACIDS IN PIGEON BREAST MUSCLE

L-Aspartic acid	654	L-Cysteine	56
L-Alanine	543	L-Histidine	50
D-Alanine	30	D-Histidine	44
DL-Alanine	434	L-Leucine	38
L-Valine	124	L-Isoleucine	24
D-Valine	53	L-Methionine	18
L-Phenylalanine	74	Glycine	0
L-Tyrosine	62		

Values expressed as μL glutamic acid formed in 40 min. Values less than 100 not considered significant by the author.

Cohn, P. P. (153).

With glutamic acid acting as donor, oxalacetic and pyruvic acids were the best acceptors, α -ketobutyric and mesoxalic acids reacted slowly and other keto acids were quite inactive. Some dibasic amino acids, such as cysteic acid, functioned in place of glutamic and aspartic acids. The outstanding reactions in order of importance were as follows (154):



Acids with amino groups other than in the α -position were not transaminated, *e.g.*, β -alanine, δ -aminocaproic, and ϵ -aminocaproic. The basic amines, such as histamine, ketones, hydroxy ketones, and aldehydes were also inactive. Peptides with free amino groups were likewise inactive. The reaction was specific for amino acids with the L configuration.

Most common inhibitors were found to have little effect on the activity of the enzyme. The most effective were benzoquinone and some metallic ions. Inhibition results at high concentrations of cyanide but not with arsenite. The action of these inhibitors may

TABLE V
RATES OF TRANSAMINATION IN VARIOUS RAT TISSUES

	Q Transamination		
	Glutamic-Aspartic	Glutamic-Alanine	Aspartic-Alanine
Heart muscle	425	7	7
Skeletal muscle	316	13	1
Brain	260	2	8
Liver	245	46	10
Kidney	245	3	3
Testis	150		
Lung	51		
Spleen	16		

Cohen, P. P., and Hekhuis, G. L. (160).

be due to reaction with the aldehyde containing prosthetic group of the enzyme (see below).

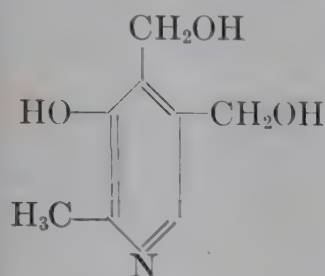
The rates of transamination in many tissues are extremely high, as shown by Cohen and Hekhuis (160) (Table V), particularly when compared with the observed rates of oxidative deamination; so that in the presence of pyruvate there may be an inhibition of oxidative deamination. Krebs had previously noted a decrease in deamination rate in the presence of various substrates which are known to form pyruvate.

The enzyme shows a different distribution from D-amino acid oxidase. It is high in skeletal muscle, liver, kidney, and brain, but is found in lesser amounts in the plant kingdom and in bacteria (161). Plants are said to carry out reactions involving aspartic acid more rapidly than those involving glutamic acid.

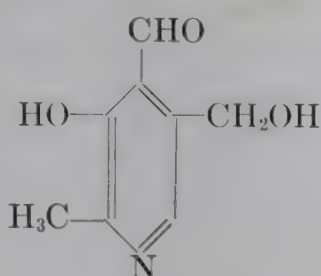
Kritzmann made a separation of two transaminating systems, and later highly purified preparations of glutamic-alanine transaminase were made by Lenàrd and Straub (388) and by Green and coworkers (270). Pure preparations of glutamic-aspartic

transaminase have also been made by the latter workers as well as by Schlenk and Fisher (519) and O'Kane and Gunsalus (471).

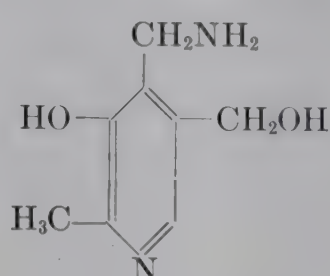
In experiments with bacteria (*Streptococcus faecalis R*), Lichstein, Gunsalus and Umbreit (402) showed that the bacteria grown on pyridoxine deficient media had greatly reduced transaminating activity. Restoration resulted upon the addition of synthetic pyridoxal phosphate.



Pyridoxine



Pyridoxal



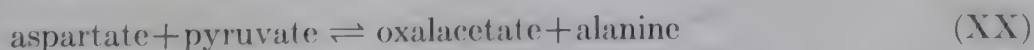
Pyridoxamine

Similarly, the transaminase activity of the tissues of pyridoxine deficient rats was reduced and could be restored, at least in part, by added pyridoxal and adenosine triphosphate (520, 16). Pyridoxal phosphate (279) was previously recognized as the coenzyme of some bacterial decarboxylases (277), and when Green and coworkers (270) assayed boiled preparations of glutamic-aspartic transaminase they found their preparations contained 0.27 μ g. pyridoxal phosphate per mg. of pure preparation. The prosthetic group of transaminase is therefore pyridoxal phosphate, but it has so far been found impossible to remove this prosthetic group without destroying the protein carrier (compare 329). The same substance is the prosthetic group of glutamic-alanine transaminase as shown by Braunstein and others (129, 263, 377).

Green and coworkers (270) question the existence of an aspartic-alanine transaminase. They attribute reaction XX to the presence of the two other transaminases, with glutamate- α -ketoglutarate acting as intermediary:



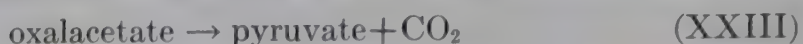
Net result:



O'Kane and Gunsalus (471) have also presented evidence that aspartic-alanine transaminase is an artifact.

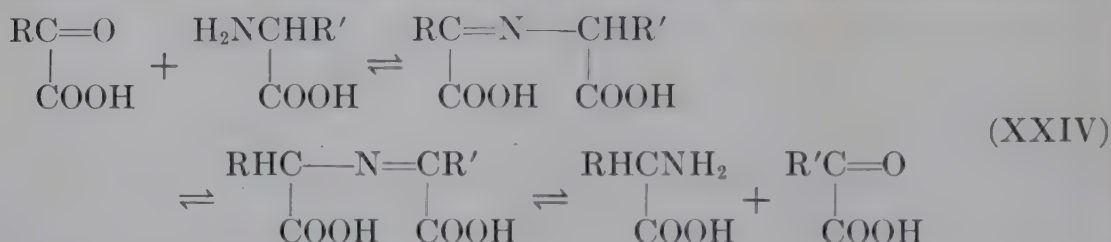
The work of Moulder, Vennesland, and Evans (448) suggests the presence of a different transaminating enzyme in pigeon liver.

It differs from the transaminases so far considered in remaining active after dialysis and also in being activated by Mn^{++} . Pigeon liver extracts incubated with pyruvate and aspartate form alanine and CO_2 without net disappearance of pyruvate. The mechanism postulated to explain these results is reaction XX followed by:



These authors make the significant suggestion that a decarboxylation reaction following transamination might favor transamination of amino acids not otherwise reactive.

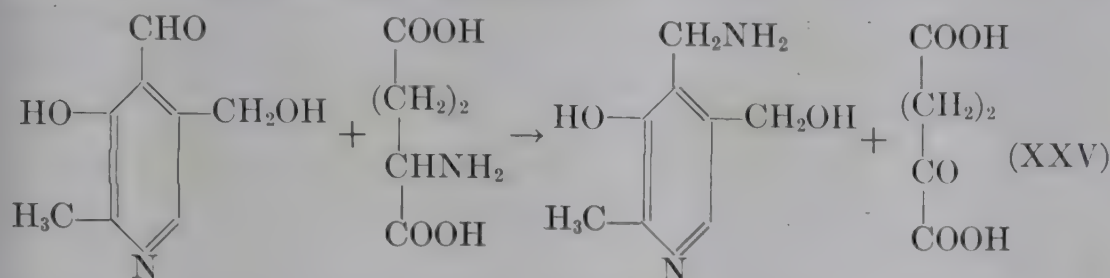
Braunstein and Kritzmann (128) proposed the following mechanism for the reaction, using two Schiff bases as intermediates:



Herbst and Engel (299) had already discovered a similar type of reaction in a pure chemical system and had postulated an analogous mechanism (297, 300). However, in the chemical system, decarboxylation of the keto acid with the production of an aldehyde took place (compare 130). Moreover, the chemical and biochemical systems differed in other respects. The most active amino acids in the chemical system were phenylglycine and its ring substitution products. Cysteine and S-ethylcysteine were also very actively transaminated with pyruvic acid, whereas glutamic acid acted slowly. In the chemical system, regardless of the nature of the starting material, the products are optically inactive, but in the biochemical system, *only* the L-amino acids are active. Moreover, in the biochemical system, the most active acids are all dibasic, and cysteine is not transaminated.

In support of the proposed mechanism (equation XXIV) Konikova and coworkers (see 124) showed that when alanine containing deuterium on the α -carbon atom was transaminated against α -ketoglutarate, the product contained no excess deuterium in the α -position. The α -hydrogen must have been lost into the water as a proton, and the new hydrogen in α -position came from the same source. Moreover, when no transamination took place, as with boiled enzyme preparations, or in the absence of α -keto acid, the α -hydrogen of glutamic acid was likewise exchanged with water (350). This suggested that there was a reaction between the amino

group and prosthetic group of the enzyme. Snell (568) showed that chemically, pyridoxal can be converted into pyridoxamine by transamination with glutamic acid:



Apparently other amino acids, notably lysine, were active, but a few were inactive, *e.g.*, proline and serine. Histidine and others were destroyed by the aldehyde.

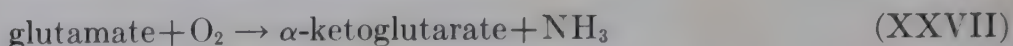
Presumably, therefore, the complete transaminating mechanism involves the transfer of the amino group first to pyridoxal phosphate, thence to the keto acid. In the experiments of Konikova and coworkers the glutamic acid formed a Schiff base with the pyridoxal phosphate and this base was capable of exchanging protons with water. The authors considered this proton exchange to be a non-specific heat resistant function of the enzyme.³ But in spite of the evidence supporting this mechanism of transamination Umbreit, O'Kane and Gunsalus (616a) have obtained data which is not easy to fit into the general picture. They found that the glutamic-aspartic enzyme from pig heart was activated with pyridoxal phosphate but not with the pyridoxamine prepared from pyridoxal phosphate and glutamic acid. The pyridoxamine was likewise inactive as the coenzyme for tyrosine decarboxylase but yet was active as the coenzyme for the glutamic-aspartic transaminase of *Streptococcus faecalis*-R. The pyridoxamine preparation obviously requires further investigation, since there may be an intermediate formed which is not activated in all systems.

6. The Relationship between Deamination and Transamination

It has already been pointed out that it is impossible to account satisfactorily for the deamination of L-amino acids in animal tissues on the basis of the L-amino acid oxidase present. Conse-

³ Attention should be drawn to this labilization of α -hydrogen in relation to experiments with deuterium, in which the presence of deuterium has been taken to indicate complete deamination followed by reamination (125). Rittenberg (505a) has also demonstrated this labilization of the α -hydrogen in L-leucine which coexists with relatively stable hydrogen atoms in the β and γ positions. Moreover, the α -hydrogens in both L-leucine and in glycine were shown to be labile relative to the amino groups of these molecules.

quently, other mechanisms of deamination have been sought. Braunstein and Bychkov (127) prepared an artificial system of the following components: transaminase, glutamic acid dehydrogenase, α -ketoglutaric acid, and pyocyanine (a dye to act as hydrogen acceptor). This was capable of deaminating L-alanine at a rapid rate through the two reactions:



More recently, Braunstein and Asarkh (126) have extended this work by showing that homogenates of rat and pig kidney deaminate

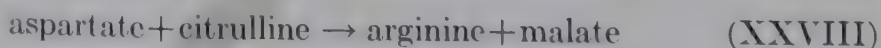
TABLE VI
AMMONIA FORMATION FROM L-AMINO ACIDS IN PIG KIDNEY
AND IN RAT KIDNEY

Amino Acid Deaminated	Homogenates of Pig Kidney				Slices of Rat Kidney
	Alone	+ α -Keto Glutarate 1-2 mg./ml.	+CoI 1-2 mg./ml.	+CoI + α -Keto Glutarate	
Glutamic acid	5.2	4.3	39.8	28.8	19.0
Aspartic acid	2.6	2.6	6.4	23.7	29.5
Alanine	1.5	2.2	5.5	26.2	19
Cysteic acid	0	0	1.5	25.9	—
Valine	1.1	1.8	1.2	10.3	7
Leucine	0.8	0.6	0.7	8.0	5
Isoleucine	—	1.0	2.3	7.1	—
Cysteine	6.0	—	—	6.2	8.2

Values expressed as μM NH_3 formed per gm. tissue in 1½–2½ hrs. in presence of 0.01 M As_2O_3 . Braunstein, A. E. (125).

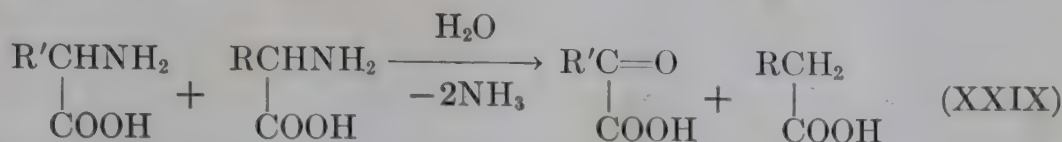
L-aspartic acid, L-alanine, L-cysteic acid, L-valine, and L-leucine only in the presence of α -ketoglutarate and coenzyme I. In the absence of the two cofactors, deamination was negligible. The results are shown in Table VI. In view of this work, it is important to determine whether amino acids in general can undergo transamination or not. Cohen (153) considered that the rates of transamination of many L-amino acids were negligible, but the rates found are not negligible if they represented real effects.

There exists a possible alternate pathway in which no free ammonia would be formed, but in which the nitrogen would appear as urea (see section VI, 1).



7. Deamination by Dismutation

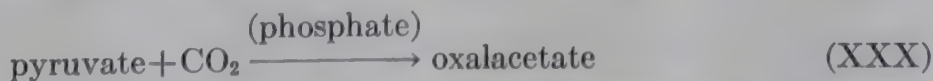
In bacteria there exists a form of coupled deamination which results in the oxidative deamination of a donor amino acid, together with the reductive deamination of a second amino acid to give a keto acid and a fatty acid according to the following scheme (588, 659, 151):



The L-isomers of alanine, valine, leucine, phenylalanine, cysteine, serine, histidine, aspartic acid, and glutamic acid can act as donors; as acceptors the following serve: glycine, proline, hydroxyproline, ornithine, and arginine.

8. Synthesis of Amino Acids

In the section on glutamic acid dehydrogenase, the reversibility of this deamination reaction has been noted. However, the synthesis of alanine, phenylalanine and tyrosine from the corresponding keto acids and ammonia was shown many years previously in perfusion studies by Embden and Schmitz (215). Neber (455) showed that amino acid could be synthesized in liver slices of guinea pigs and cats from pyruvate and ammonia, part of the ammonia being converted at the same time to urea. At low concentrations of pyruvate the synthesis of urea proceeded more rapidly than that of amino acid. Tissues taken from fasted animals showed a reduced capacity for both the synthesis of urea and of amino acid. The nature of the mechanism has been investigated extensively by Kritzmann (376) in both rat liver slices and in homogenates and cell free extracts supplemented with CoI, the transaminase coenzyme and fumarate. It was found that the synthesis from added pyruvate did not proceed in the absence of bicarbonate and phosphate. Since it is known that phosphate is necessary for carbon dioxide fixation (618), these results were interpreted as showing that oxalacetate is an essential component of the system and that aspartic acid, formed from this keto acid, acts as an amino group carrier to the pyruvate as follows:



Transamination with pyruvate (reaction XX) then completes the synthesis of alanine. In agreement with these assumptions it was shown that amino acid synthesis proceeded in slices in the absence of CO_2 and phosphate if oxalacetate or another member of the tricarboxylic acid cycle were present. Moreover, during the synthesis from pyruvate in the presence of fumarate there was first a formation of aspartic acid which later was converted to alanine. The total amino-nitrogen formed was accounted for in the forms mentioned; little glutamic acid accumulated. However, the intermediary formation of the C_5 acid was not excluded, and in view of the existence of an active system for the amination of α -ketoglutarate a primary formation of the corresponding amino acid would have been anticipated. The evidence from isotope studies indicates that the primary product is glutamic acid rather than aspartic acid (see section VII). Moreover, as previously mentioned reaction XX proceeds less readily than other known reactions of the same type.

It should be noted that Wiss (653b) found that he is unable to confirm the findings of Kritzmann. This worker using different methods, obtained a synthesis of alanine in concentrated homogenates of both rat and guinea pig liver. Evidently the synthetic system resides in particles which can be sedimented from the homogenate. During the synthesis he was unable to demonstrate any accumulation of aspartic acid, although Kritzmann only appears to have found accumulation of this amino acid when some dicarboxylic acid such as fumaric, was added to the system. The lack of accumulation of this substance in the Wiss experiments is no proof that it is not an intermediate. Wiss showed that synthesis was inhibited in the absence of oxygen, or when cyanide and arsenite were added.

Quastel and Woolf and others (488, 166, 256, 654) showed that in preparations of resting bacteria an amino acid can be synthesized by another mechanism:



The reaction has been investigated from the thermodynamic point of view by Borsook and Huffman (96, 110) who showed that the ΔF for the reaction is -3.72 Cals., when calculated from equilibrium data. When calculated from thermal data a very similar value was

⁴ The amination goes so well that the method may even be of significance in the preparative field. From 3 gms. of fumaric acid they isolated 3.5 gms. of copper aspartate (488).

found. In contrast to this the ΔF for the reaction involved in the synthesis of alanine from ammonium-ion and pyruvate was calculated to be +39.5 Cals. This reaction can, therefore, by no means proceed spontaneously.

9. The Asymmetric Nature of Amino Acid Synthesis

Various authors have supposed that a mixture of the optical isomers is produced when amino acid is synthesized within the organism. It has already been noted that glutamic acid dehydrogenase and transaminase, enzymes which are intimately concerned with the synthesis, are both highly specific for the natural forms. Further proof that the synthesis proceeds with the formation of the L-isomer only has been provided by the work of Shemin and Rittenberg (546).

When large doses of DL-tyrosine are fed to rats, an excretion of the D-form is observed. Also, if ammonia containing isotopic N is fed, the isotope appears in the amino group of all the amino acids with the exception of lysine, *i.e.*, synthesis of amino acid takes place. So if both tyrosine and labeled ammonia are fed, the excreted D-tyrosine is found to contain no excess amount of the isotope. Therefore, no D-tyrosine synthesis occurred.

Experiments of a slightly different type were done with glutamic acid. D-glutamic acid containing isotopic nitrogen was fed to rats. The amino acid appeared in the urine without having suffered any dilution of its isotope content. These experiments conclusively show that neither D-glutamic acid nor D-tyrosine is synthesized by the rat in anything but trace amounts. It is evident that the synthesis of amino acid proceeds asymmetrically in the organism to produce the L-form.

III. DECARBOXYLATION OF AMINO ACIDS (72, 259, 307)

Under the influence of the appropriate enzymes, the decarboxylation of various amino acids can occur in animal tissues. Among these may be mentioned histidine, tyrosine, tryptophan, dopa (3,4-dihydroxyphenylalanine), and cysteic acid. Of the amino acids mentioned the one most actively decarboxylated is dopa.⁵

⁵ The amines produced in the reaction are usually physiologically active, and their presence is conveniently detected by their effect on the blood pressure of suitable animal preparations. However, it is necessary, in those cases where negative results are obtained, to ascertain that the amine is not being destroyed as rapidly as it is formed. In the case of dopa decarboxylase the enzyme activity is such that the decarboxylation can be followed by measuring the evolution of carbon dioxide.

The enzymes are specific for the L-forms and for the specific amino acids except that the tyrosine and tryptophan enzymes may be the same. The enzyme attacking histidine is inhibited by the D-isomer and also by dopa, adrenaline, and cyanide as well as by carbonyl reagents such as hydroxylamine and semicarbazide. Fed or injected dopa is partly excreted in the urine as hydroxytyramine (in conjugated form) (308). The dopa enzyme may be important in the formation of adrenaline. The enzyme responsible for the decarboxylation of cysteic acid results in the production of taurine. Homocysteic acid is not decarboxylated.

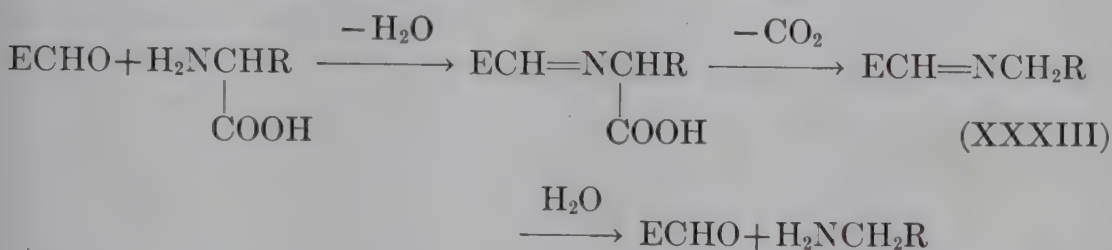
The relative rates at which amino acids are decarboxylated varies with the species. Generally, the kidney contains the most active enzyme with smaller amounts being present in liver, intestine, and pancreas. It does not seem possible that the pathway through decarboxylation is the main one for the breakdown of the amino acids mentioned in higher animals.

Amine production in bacteria was first studied by Ellinger (210) and by Ackermann (5). All the amino acids which undergo decarboxylation contain additional polar groups, although phenylalanine is decarboxylated (163). Many species of bacteria are capable of forming histamine, but the formation of other amines is more restricted. In the absence of the appropriate substrate, the bacteria do not form the specific decarboxylase except in the case of glutamic acid decarboxylase. Koessler and Hanke (347, 287) supposed that the amine formation was a response to acidification of the medium, that is, the amine formation was considered to be a protective mechanism.

These decarboxylases are also specific for the individual L-acids (257, 218). Six have been prepared relatively pure, and such preparations can be used to advantage in the determination of amino acids by following the reaction manometrically (258).⁶ The pH optima for these enzymes are all on the acid side of pH 6 and the enzymes are sensitive to heavy metal ions such as Ag^+ and Hg^{++} and cyanide, but less sensitive to azide. They are inhibited particularly by carbonyl reagents such as hydroxylamine, hydrazine, and semicarbazide (604), with the enzyme decarboxylating glutamic acid providing an exception to this generalization (261). The inhibiting action of these reagents suggests that these enzymes have a carbonyl radicle in the prosthetic group.

⁶ Neuberger and Sanger (467) have used a lysine decarboxylase from bacteria in the preparation of D-lysine, obtaining a yield of 45% of the theoretical from 2 gm. of the DL-mixture.

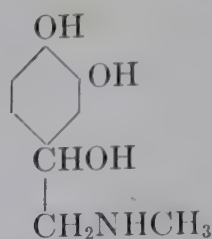
Bellamy and Gunsalus and others (44, 42, 277, 616) found that *Streptococcus faecalis* grown in a simplified medium could not be adapted to decarboxylate tyrosine at a significant rate unless pyridoxine and nicotinic acid were present. In other species these cofactors have no effect on the development of the enzyme. Pyridoxal and pyridoxamine were later shown to be more effective than pyridoxine in some species (569) and in dried bacterial preparations, pyridoxal phosphate is the only effective agent (278). A coenzyme can be prepared from the protein part of the isolated decarboxylase by precipitating the protein in alkaline solution (except for histidine and glutamic acid decarboxylases) (645), and the coenzyme is pyridoxal phosphate (27, 330, 516). Since the free aldehyde group is necessary for the action of the enzyme, the mechanism of decarboxylation may be pictured as follows (645):



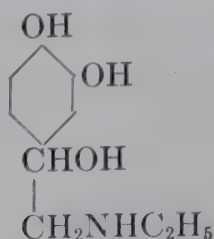
IV. OXIDATION OF AMINES

1. Amine Oxidase

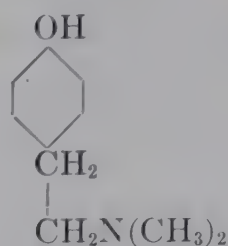
Amine oxidase brings about the oxidation of a large variety of amines such as tyramine, adrenaline, and aliphatic amines. Generally, there is most enzyme in the liver, but brain and kidney are also active (486, 63). Ewins and Laidlaw (223) observed the oxidation of tyramine to *p*-hydroxyphenylacetic acid in liver perfusion trials. The studies of Philpot (482) show that the primary oxidation product is *p*-hydroxyphenyl acetaldehyde. In addition, there is a production of hydrogen peroxide (349). Either oxygen or some dye such as *o*-cresol indophenol acts as hydrogen acceptor. The oxidase is not inhibited by cyanide, in which respect its behavior is in contrast to that of diamine oxidase. Adrenaline (I) is also oxidized with the production of methyl amine and the corresponding ethyl substituted amine (II) produces ethyl amine. Hordenine (III) is oxidized, giving dimethyl amine (501). Consequently, if this deamination proceeds via dehydrogenation like those already considered, it must be possible to dehydrogenate the ionized form (ammonium). In the case of amino acids this apparently does not occur.



I. Adrenaline



II.



III. Hordenine

In view of the above findings the reaction may be formulated as follows:



Further oxidation of the aldehyde readily occurs in the absence of cyanide, so that in brei experiments more oxygen may be consumed than is required to satisfy equation XXXIV (289). In muscle tissue, in addition to oxidation of the amino group, there is also a rupture of the aromatic ring (59).

The enzyme oxidizing adrenaline is identical with that for tyramine and the aliphatic amines (75), but it is possible that there are several oxidases because some amines behave atypically (56). Amines with short chains are oxidized slowly and those with long chains (C_{18}) are not oxidized (76). Some diamines with long chains are oxidized by the amine oxidase (74). Adrenaline is oxidized more slowly than tyramine, and the D-form more slowly than the L (76). In fact, the rate of oxidation of adrenaline is so low that a large amount of the compound is excreted as a conjugate rather than as an oxidation product (502). Amidines inhibit these oxidations (73, 294).

2. Diamine Oxidase (667)

The diamine oxidase is specific for diamines or amines with a second basic group in the molecule, *e.g.*, imidazole or guanidine. The enzyme was first called histaminase by its discoverer, Best (62), although it has been shown since to be less specific than originally supposed (668). It attacks putrescine, cadaverine, and agmatine, the amines corresponding to ornithine, lysine and arginine. The mechanism of enzyme action is like that of amine oxidase, ammonia and hydrogen peroxide being produced. Oxidation is inhibited by cyanide, choline, thiamine, and various guanidines. It is also inhibited, like decarboxylase, with aldehyde reagents.

The enzyme is present in liver, kidney, intestinal mucosa, pros-

tate, and seminal vesicles. In pregnancy there is a considerable rise in the enzyme in the serum, which may represent diffusion out of the placenta (11).

V. REACTIONS INVOLVING THE AMINO GROUP (553, 664, 80, 649a)

1. Acylation of Glycine

As early as 1829 Liebig had found hippuric acid (benzoyl glycine), which is a normal urine constituent in herbivora, in the urine of the horse. It is not formed in birds and reptiles. Using the excretion of this substance as an index, Abderhalden and Hirsch (3) showed that glycine could be synthesized in the body. The rate of formation of glycine for this purpose may be very high. The estimates of Lewis (399) are given in Table VII.

TABLE VII
RATE OF GLYCINE SYNTHESIS FOR HIPPURIC ACID FORMATION

	mg./kg./hr.
Dog	3.5
Man	9.0
Pig	15.0
Rabbit	25.0

Lewis, H. B. (399).

The requisite nitrogen for the hippuric acid may come from that diverted from urea formation (397), although excessive doses of benzoic acid lead to the breakdown of body protein. Schoenheimer (524, 506) has also shown, using glycine labeled with N^{15} , that most of the glycine for hippuric acid formation is of endogenous origin. Glycine arises to some extent from the breakdown of other amino acids, primarily of serine (545), as shown by the data in Table VIII which gives an indication of the readiness with which the nitrogen in various compounds is transferred to form hippuric acid. The experimental animals were first fasted, then were fed a standard dose comprising equal moles ($0.35mM$) of the nitrogen compound and of benzoic acid. It will be observed from the table that very different degrees of dilution were observed, least after glycine, then a greater degree after L-serine, L-glutamic acid, with the rest suffering much greater dilutions still. With the D-amino acids the tremendous dilutions observed indicate that they must have suffered complete deamination prior to nitrogen transfer to glycine, and since the dilutions are generally higher than observed

after feeding ammonia, either a lack of catabolism or loss of unchanged compound into the urine is indicated (section X, 2, 4). The small dilution suffered by L-serine nitrogen either indicates a direct transfer of the nitrogen to glycine or a conversion of this

TABLE VIII
INCORPORATION OF N¹⁵ FROM VARIOUS SOURCES INTO HIPPURIC ACID

	Dilution*	
	Rat	Guinea Pig
Ammonia (Av. 2 expts.)	395	21
L-glutamic acid	45	10
D-glutamic acid	1500	450
Glycine	2.8	2.4
L-serine	5.5	3.9
D-serine	158	145
L-alanine	94	21
L-leucine	120	54

* Dilution = At. % excess in compound fed / At. % excess in hippuric acid isolated.
Shemin 1D. (545).

amino acid to glycine. It appears from other data that the former is the correct interpretation (section X, 4, II, 4b), but in the case of glutamic acid it is impossible to make a choice between these two possibilities.

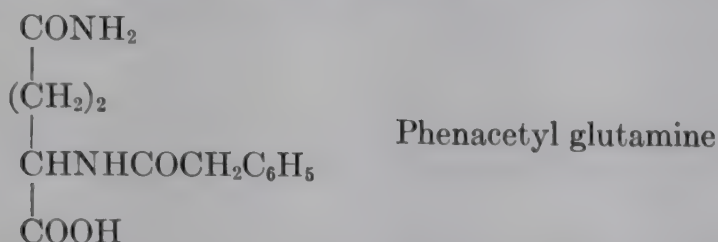
It should be remarked in connection with this work that, as recognized by Shemin, no estimate can be made from the above data concerning the normal rate of conversion of glycine to serine, since the conversion process presumably is being stimulated by the benzoic acid fed, and since there may be independent pathways of serine synthesis which are being interfered with under these experimental conditions. Also, unless the yield of hippuric acid is the same in all the experiments, no strict comparison can be made between the results of feeding one or another substance.

As first shown by Jaffé (318) another acid, phenylacetic acid, is similarly conjugated with glycine in several species, *e.g.*, dogs and rabbits, and nicotinic acid also is conjugated with glycine in the rat, dog and man although part of the substance is methylated (518).

These conjugations with glycine take place in the liver and the formation of hippuric acid after a dose of sodium benzoate has been used as a liver function test (489). Other compounds, such as *p*-aminobenzoic acid are similarly conjugated and further consideration will be given to these reactions in section 5.

2. Acylation of Glutamine

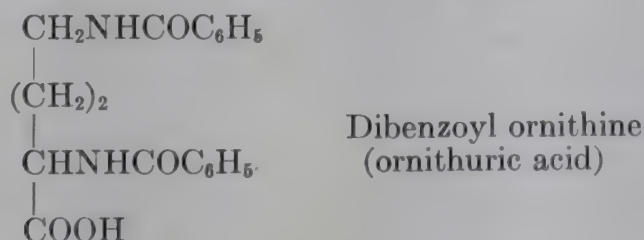
In man and the higher apes phenylacetic acid, instead of being conjugated with glycine, is coupled with glutamine, the product being phenacetyl glutamine (485, 554, 610):



Benzoic acid, on the other hand, is conjugated with glycine to give the more usual type of product. Thus, in the human, both glycine and glutamine are produced in quantity and at the same time when both phenylacetic and benzoic acids are given (556). The synthesis proceeds at the expense of nitrogen which would otherwise appear in the urine as urea. When the amino group is blocked with the phenacetyl group the further metabolism of the amino acid cannot proceed (557). Surprisingly enough, *o*-chlorophenylacetic acid is conjugated with glycine in the human (147), and no glutamine derivative is formed.

3. Acylation of Ornithine

A third amino acid, ornithine, is conjugated in the fowl with phenylacetic (615) and benzoic acid, as first shown by Jaffé (318, 173). The derivative contains two acyl groups:

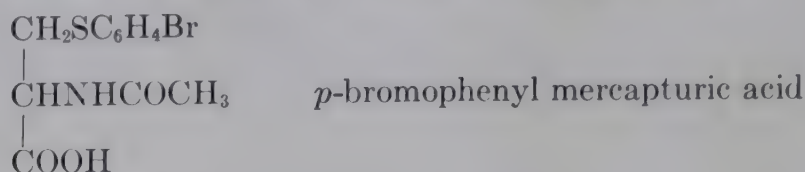


The acyl derivative is synthesized at the expense of nitrogen, which would otherwise appear as uric acid in the excreta (174). Even if a fowl is given extra glycine along with the benzoic acid, the two are not conjugated (663).

4. Acetylation of Cysteine

Many years ago Jaffé (319) and Baumann and Preusse (39) observed that after feeding bromobenzene or other analogous aromatic halides, dogs excreted a sulfur compound in the urine

This compound was shown to be an acetylated derivative of cysteine and was named a "mercapturic acid," *e.g.*,



Feeding chloro- or iodobenzene (40) leads to the excretion of similar derivatives, and indeed a large group of aromatic compounds such as benzene (144, 666), naphthalene (116, 578), anthracene (118), and fluorobenzene (167, 665) when fed are in part similarly conjugated (579) and thus lead to an increase in the neutral sulfur fraction of the urine; that is, the fraction of the urine sulfur which is not in the form of sulfate even after hydrolysis. The conjugation does not take place in the dog only, but is found in the cat, rabbit, rat, mouse, pig and man. Stekol has shown that cystine and methionine added to the diet increase the yield of mercapturic acid. (581).⁷

Although the mechanism of the reaction has not been elucidated, it has been shown that phenol is not an intermediate in mercapturic acid formation (168). Furthermore, the acetylation will take place following the coupling of the aromatic compound with the sulfur of cysteine. Thus, Stekol (582) observed that after feeding S-benzylcysteine or S-benzylhomocysteine, the corresponding acetyl derivatives were excreted. The D-isomers of *p*-bromophenylcysteine and of S-benzylcysteine are directly acetylated with consequent excretion of the D-derivative in the urine (68, 628, 583). So, although acetylation of D-amino acids was not observed by Knoop and coworkers it occurs.

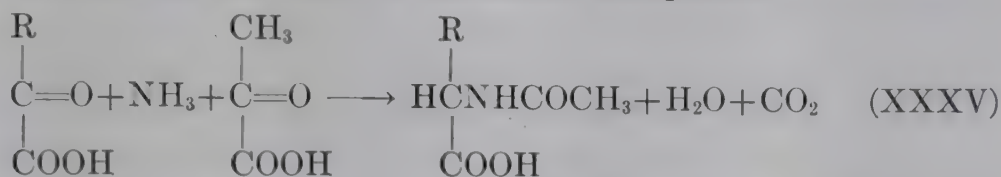
Unlike the conjugations so far considered in which the amino acids necessary are synthesized by the organism at the expense of nitrogen which would otherwise have been excreted as urea or uric acid, the sulfur for the synthesis of mercapturic acid cannot (always) be provided by diversion of sulfur from sulfate formation. The requisite cystine is either provided from dietary cystine or methionine or from the breakdown of body tissue (449).

5. Acetylation of Foreign Amines

Many foreign amines, when fed, are excreted to a greater or lesser extent in the acetylated form, *e.g.*, sulfanilamide and *p*-aminobenzoic acid. Knoop attached considerable importance to the acetyla-

⁷ A method of determining mercapturic acids has been described by Stekol (580).

tion he observed in the dog fed γ -phenyl- α -ketobutyric acid because it demonstrated the synthesis of an amino acid from a keto acid, even when the keto acid provided was presumably a foreign substance. He proposed an acetylating mechanism in which pyruvate became the source of the acetyl group since fed pyruvate appeared to increase the yield of the acetyl compound.



de Jong (324) and Erlenmeyer and Kunlin (219) had previously observed that pyruvic acid in the presence of ammonium salts condensed to form acetylalanine.

However, due to misinterpretation of observations made after feeding the acetyl derivatives of DL-phenylalanine and DL-phenylaminobutyric acid, Knoop and Blanko (344) abandoned the pyruvate mechanism because the acetyl compounds excreted had levo rotation and *were assumed* to have the same configuration as the natural amino acids. They recognized that the L-acetylamino acid should have been metabolized more readily than the D-acid by deacylation, deamination and oxidation to benzoic acid or reamination to the L-acid. Actually, the excreted acetyl compounds belong to the D series as shown by du Vigneaud and Irish (626). On feeding D-phenylaminobutyric acid to a dog, these authors observed the excretion of the L-acetyl derivative. Thus, the optical inversion of the foreign amino acid was shown. The results are summarized in the following table:

<i>Fed</i>	<i>Excreted</i>
(1) D-aminophenylbutyric acid	D-aminophenylbutyric acid L-acetylamino-phenylbutyric acid
(2) L-aminophenylbutyric acid	L-acetylamino-phenylbutyric acid
(3) ketophenylbutyric acid	L-acetylamino-phenylbutyric acid
(4) DL-acetylamino-phenylbutyric acid	D-acetylamino-phenylbutyric acid

Why no L-acetylamino acid appeared in the urine when the DL-mixture was fed is not entirely clear. In the case of the essential amino acids, the acetyl derivatives replace the free acids in the diet; they are hydrolyzed and possibly represent intermediates in the synthesis of amino acids. Bloch and Borek (82) have observed the acetylation of three natural amino acids with deuterium labeled acetate in liver tissue slice experiments.

Du Vigneaud and coworkers (623) prepared both stereoisomers of phenylaminobutyric acid labeled with N¹⁵ and fed these amino acids to rats whose body fluids were enriched with D₂O. After

feeding the D-isomer the acetylated product was found to contain no N¹⁵ but had deuterium, presumably on the α -carbon, whereas after feeding the L-isomer, the acetyl derivative contained both D and N¹⁵. Thus in order to form the acetylamino acid from the D-compound, the amino acid is first deaminated, then reaminated. Whether the reamination proceeds by the pyruvic acid mechanism is not shown by these experiments. The replacement of the α -hydrogen by deuterium from the body water might have resulted from the amino acid-imino acid equilibrium, or due to the formation of some Schiff base containing labile hydrogen. Fishman and Cohn (237) later found that the acetyl group also contained deuterium. Thus the "stable" hydrogen of the acetylating agent is in equilibrium with the body water. This may be accounted for if the acetylating agent is formed from acetoacetate or pyruvate. It should be added that when the acetylamino acid itself is fed (237) no deuterium is introduced into the molecule.

The existence of a mechanism for the direct acetylation with acetic acid has been shown by Bernhard (51) who studied the acetylation of sulfanilamide, *p*-aminobenzoic acid and hexahydrophenylalanine by giving acetate labeled with deuterium to experimental animals. Deuterium appeared in the products but in a much diluted state, showing that there was a large production of acetate within the organism or labilization of its hydrogen. Other possible sources of acetate have been examined by Bloch and Rittenberg (83) by feeding other deuterium labeled compounds along with phenylaminobutyric acid. The following substances gave rise to acetyl groups: alcohol, butyric acid, alanine, valeric acid, and myristic acid; but no acetate was formed from propionic acid. Again, acetate was shown to function directly as an acetylating agent (85, 52). An attempt was also made to answer the question concerning the existence of a pyruvate mechanism by feeding alanine labeled with deuterium, the alanine acting as a source of pyruvate. The results of these experiments are summarized in Table IX.

With acetate the concentration of D in the product is proportional to the amount of acetate fed and independent of the amount and nature of the foreign amine (Table IX, col. 3). With alanine the concentration of D in the product varied with the nature of the substance acetylated. The aromatic amines appeared with low concentrations of D in the product, the phenylaminobutyric acid with high concentrations. This was taken to mean that the aromatic amines were directly acetylated with the acyl group from

TABLE IX
ACETYLATION OF AMINO COMPOUNDS WITH DEUTERIUM
LABELED ACETATE OR ALANINE

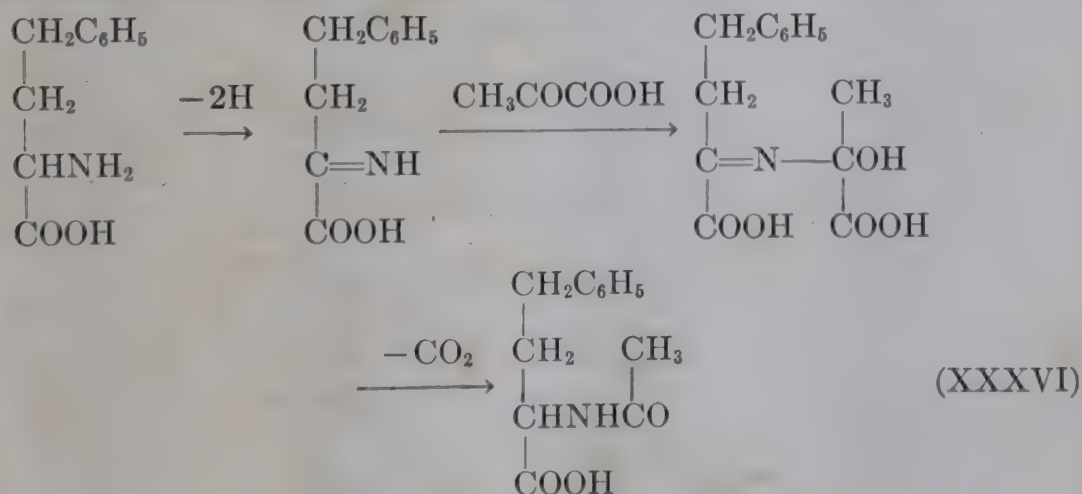
	Acetate Fed <i>mM.</i>	Compd. Fed <i>mM.</i>	D in Acetyl Amino Deriv. Excreted*	Alanine Fed <i>mM.</i>	Compd. Fed <i>mM.</i>	D in Acetyl Amino Deriv. Excreted*
DL-phenyl amino- butyric acid	0.078 0.81 1.52 1.60 1.68	0.45 0.10 0.55 0.55 0.44	4.3 4.3 4.7 5.3 5.3	0.84	0.55	3.2
L-phenyl amino- butyric acid	1.60	—	5.1			
D-phenyl amino- butyric acid	1.51	—	5.4			
<i>p</i> -amino benzoic acid	1.57	—	5.7	1.55 0.81	0.73 0.73	0.8 0.4
Sulfanilamide	0.91	—	6.0	0.91	0.40	0.6

* These data are the average of results from two or three rats, calculated to the basis of 100 at. percent excess D in the methyl group of the compound fed, and for a dosage of one *mM* of the deutero compound per 100 gm. of rat. i.e.,

$$\frac{\text{At. \% excess in acetylated compd.} \times 100.}{\text{At. \% excess in compd. fed} \times mM}$$

Recalculated from data of Block, K., and Rittenberg, D. (85).

acetic acid, whereas the phenylaminobutyric acid was partly acetylated via a pyruvate mechanism. Obviously, the aromatic amines, unlike the aliphatic amines, cannot be converted to the necessary imino intermediate to allow of a pyruvate mechanism:

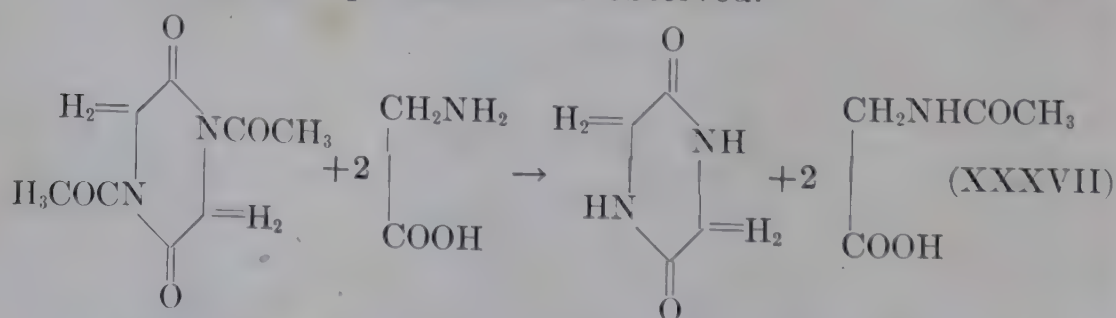


However, the existing experimental data do not prove the existence of a pyruvate mechanism. The yield of acetylated product from phenylaminobutyric acid is very much greater than from the aromatic amines, consequently the demands placed on the acetylat-

ing mechanism in the one case are very much greater than in the other. Thus, the demand for acetylating agent may result in a more rapid formation of the agent from pyruvate than is possible from acetate. If the pyruvate is labeled with deuterium, then the introduction of D will be greater in the case of substances readily acetylated than in the case of those less readily acetylated. At least the results allow no other interpretation than that the direct acetylation is of more importance than the indirect. However, it should be noted that the pyruvate mechanism does not require any special acetylating agent other than this ubiquitous substance and is one that may be expected to proceed spontaneously due to the oxidation involved.

The existence of another type of acetylation has been indicated by Bloch and Rittenberg (86), who showed that the acetyl derivatives of glycine, L-alanine, L-glutamic acid, and L-leucine bring about the acetylation of phenylaminobutyric acid, presumably by a direct transfer of the acyl group (transacetylation). Such transfers are not possible from the acetylated D-amino acids, nor do the aromatic amines function as acceptors. Rather unexpectedly, the acetyl group is transferred from the ϵ -amino group of lysine. It should be noted that this derivative of lysine, in contrast to the α -acetyl derivative, supports growth of rats deficient in this amino acid (467). The interpretation of these data depend on the possibility of forming an intermediate acetylating agent from the derivatives in question. Should this be possible, no transacetylating mechanism need be postulated to explain the results.

However, transfers of acetyl groups have been observed in a purely chemical system by Bergmann and coworkers (49). On heating diacetyldiketopiperazine with an alkaline solution of glycine, an acetyl group transfer was observed.



The same type of reaction proceeded with the analogous derivative of arginine without the addition of base.

It is quite obvious that these acylations resemble in many re-

spects the synthesis of peptide bonds. Borsook and Dubnoff (98) investigated the synthesis of hippuric acid in liver tissue slices from this point of view and showed that the synthesis cannot be considered as the reverse of hydrolysis because of the energetic requirements. Synthesis must be coupled with an energy yielding reaction. It cannot proceed when progress of the energy yielding reactions is inhibited. Hippuric acid synthesis is relatively slow compared with the rate of synthesis of urea and the rate falls off considerably as the slices age. More recently, synthesis has been attained in homogenates of liver primed with α -ketoglutarate and adenylic acid (104). With these substances present the homogenate in oxygen presumably forms adenosine triphosphate, which acts as the energy donor.

A similar system has been studied by Cohen and McGilvery (162), who used, in place of benzoic acid, the *p*-amino derivative. The previous findings were confirmed by showing that the energy requirements must be supplied by an oxidative system or by adding adenosine triphosphate. The formation of *p*-aminohippuric acid was inhibited by cyanide, arsenite, iodoacetate, fluoride and Ca^{++} . Less inhibition was observed with azide and malonate. Under the best conditions the rates of synthesis were similar to those observed by the previous workers. In kidney the values proved to be higher but there was no detectable synthesis in testes, muscle, brain or spleen. The synthetic system was associated with the material sedimented from homogenates and it was stimulated by the dicarboxylic acids of the tricarboxylic acid cycle as well as by citric and pyruvic acids and by K^+ and Mg^{++} . There was no stimulation by adenosine triphosphate and coenzyme I alone, but with simultaneous addition of fumarate, a very active system resulted. The enzyme system was unstable to freezing, acetone drying, and to hypo- and hypertonic solutions. No synthesis took place from acetylglycine or glyoxalic acid, so the mechanism involved the amino acid or some nitrogen-containing derivative.

The relation of hippuricase (522), which can bring about the hydrolysis of hippuric acid, to the synthetic system is not known. This enzyme is widely distributed in bacteria and moulds as well as in most animal tissues. It will hydrolyze many ring substituted hippuric acids (212) although hippuric acid when fed is excreted without undergoing hydrolysis (175).

Another analogous reaction which has been studied extensively is the acetylation of sulfanilamide which occurs in most species except the dog (422, 254). In liver slices this foreign amine was more readily acetylated than *p*-aminobenzoic acid, sulfathia-

zole or sulfadiazine (340). The acetylation did not proceed in homogenates unless these were carefully prepared. The activity was increased by adding acetate, but acetyl phosphate did not exert any specific effect on the conjugation. However, oxygen was not necessary if adenosine triphosphate were provided (407). Inhibition resulted when hydroxylamine was added, which presumably indicated the participation of acetyl phosphate or other carboxylphosphate in the system, because hydroxylamine reacts more or less specifically with such phosphates (410).



A heat stable, water soluble coenzyme is one component of the system (408). This coenzyme contains pantothenic acid (409) and it is subject to acetylation.

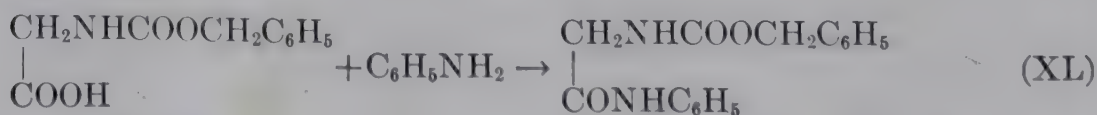
6. Peptide Bond Formation (638, 470, 97a)

The most obvious problem with regard to the synthesis of protein concerns the mechanism of peptide bond formation. Formerly, it was thought that this was a matter of reversing the enzymic digestion of protein. This was first attempted by Danilewski and many others continued this type of work with varying success. Wastenays and Borsook (638) have reviewed the older studies. It depends for its success on reducing the concentration of water to such an extent as to shift the equilibrium:



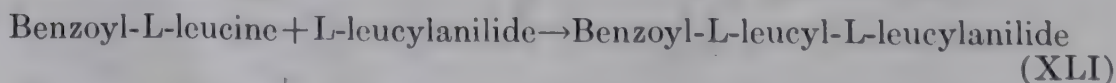
in favor of synthesis. From the concentrated products of the partial hydrolysis of protein there were formed insoluble protein-like materials called plasteins. Sometimes decreases in amino nitrogen were observed (165), but the conditions used in these experiments depart so widely from those under which protein synthesis proceeds in living matter that it is hardly possible that synthesis takes place in this way.

More recently, Bergmann and coworkers (50) have observed the synthesis of insoluble peptides in simple systems such as the following:



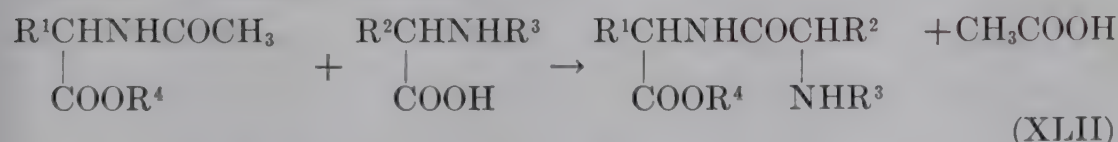
Carbobenzoxycysteine

Carbobenzoxycysteine-anilide

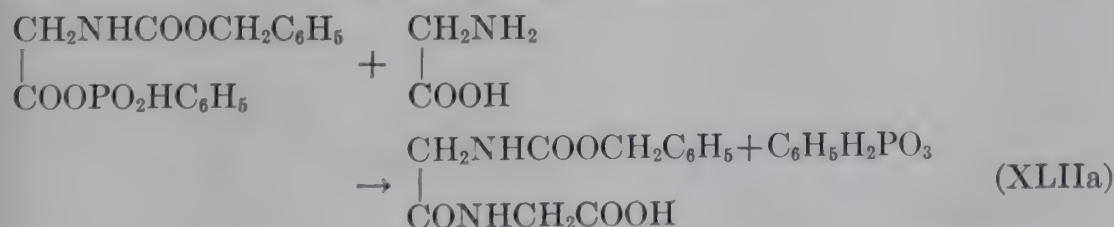


The syntheses which occurred in the two examples given were catalyzed by papain. Other similar syntheses proceeded in the presence of proteolytic enzymes such as bromelin and cathepsin.⁸ It is quite obvious that such syntheses as these represent very special cases in which product insolubility provides the necessary driving force. Most peptides are more soluble so that these examples do not provide a general solution to the problem of peptide bond synthesis.

Since the direct synthesis of peptide bonds requires energy, many indirect mechanisms have been proposed, *e.g.*, syntheses through keto acids (301), aldehydes (404), acetyl amino acids (508). Keto acids and aldehydes can hardly be the intermediates because when nitrogen labeled amino acids are fed, the highest concentration of isotope in the proteins is always found in the initially labeled amino acid. Consequently, it is impossible that an N-free derivative serves as an intermediate. As previously mentioned, acetylglycine does not promote the formation of *p*-amino hippuric acid; likewise, it is no better than free glycine in the synthesis of glutathione (81). It, therefore, appears that the acetyl mechanism proposed by Rittenberg and Shemin (508) is not operative (reaction XLII).

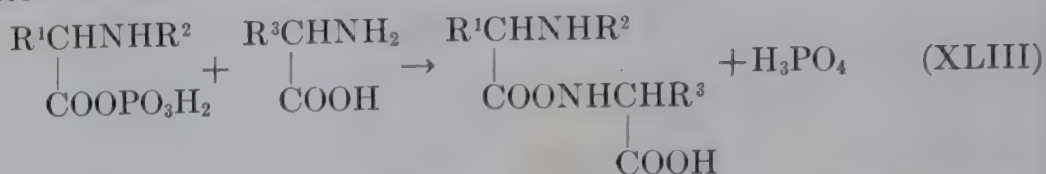


However, in this connection it should be noted that dibenzoyl phosphate reacts chemically with glycine to form hippuric acid at a rapid rate (149). Similarly, the same worker, Chantrenne (149a) has shown the carbobenzoxyglycylphenylphosphate reacts with glycine at room temperature and at neutral pH to produce the substituted dipeptide and phenylphosphate.



⁸ The specificity of the proteolytic enzymes is such that it provides a method of separating the isomers of amino acids (253). Activated papain will catalyze the reaction between benzoylleucine and aniline only when the leucine is of the L configuration, so from a DL-mixture of benzoylleucine, the D component will not form the anilide and will remain in solution, whereas the L component will precipitate as the anilide. The method is quite generally applicable. Using the same principles it has proved possible to separate the optical isomers of methionine (184).

It is possible, according to Chantrenne, to synthesize the substituted peptides of glycylglycine, glycyltryptophan, and glycylglycyltryptophan, and in this manner to produce a very large variety of peptides of varying complexity. Lipmann (406) has proposed a scheme in which the active agent is a carboxylphosphate:



It is obvious the protein synthesis involves more than has been indicated so far, and according to Lipmann (407a) it may conveniently be divided into two parts, the one of peptide bond synthesis, essentially *Polymerization* and the second one of *Patternization*, that is one of obtaining a synthetic product with the same amino acid composition and arrangement as occurs in some particular protein or protein fragment. However, it might be better to reserve the term polymerization for the final stage in the synthesis and consider the process as one occurring in three stages viz, peptidization, patternization and polymerization where the last term denotes the process whereby relatively large peptides are condensed and sterically oriented to form the protein structure. From what has been said with respect to glutamine synthesis and the formation of various acylated compounds, and from what follows on glutathione, it has become fairly clear how the process of peptidization must occur.

The patternization problem arises first when the formation of a tripeptide such as glutathione is examined. How is the synthesis organized to produce this combination of three amino acids—the one with its δ -carboxyl group in peptide linkage? Bloch and coworkers (83, 323a) have begun an investigation of this synthesis using glycine- N^{15} and C^{14} . The carrier technique was used to facilitate the glutathione isolation. It was shown that synthesis will proceed in extracts of acetone dried pigeon livers as well as in homogenates of this tissue. The synthesis goes only in the presence of the requisite amino acids, and of adenosine triphosphate in low concentration. Acetyl glycine is less effective than free glycine, particularly in the extracts, and glutamine is not so well utilized as glutamic acid. Synthesis proceeds more readily aerobically and accordingly it was found to be inhibited by malonate and by dinitrophenol. The extracts are also rich in glutamic dehydrogenase so

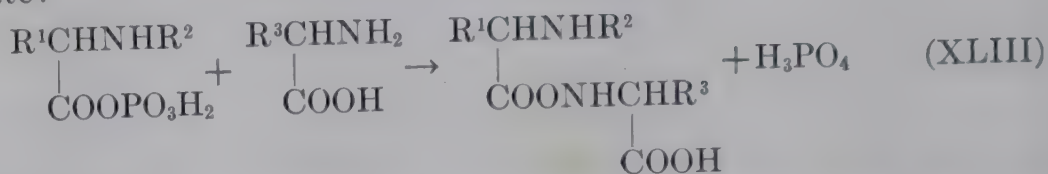
that if labeled ammonia is added to the preparation it becomes incorporated in the glutamic acid moiety of the molecule; so the rate of incorporation of glutamic acid- N^{15} cannot be measured because of the interference from the equilibration of its nitrogen with the free ammonia appearing in the system. It would be of interest with respect to the patternization problem to determine the relative rates of incorporation of cystine, glycine and of glutamic acid (using chain labeling) under different conditions to see if the synthesis proceeds as a unitary process or if one or other or both of the possible dipeptides are involved as intermediates. The actual rate of incorporation of glycine was 0.4–0.9 micromoles per hour per gram of liver. This may well have represented accumulation because apparently there was no difference in the rate whether carrier glutathione was added before or after the incubation.

To date the synthesis of peptides other than glutathione has not been investigated, and attention has been devoted to the problem of the synthesis of the protein molecule as a whole. Work of this type was initiated by Melchior and Tarver (436) who showed that it is possible to detect the uptake of labeled methionine by the proteins of liver slices incubated in saline solution for a period of a few hours. Similar data was also provided by Frantz, Loftfield and Miller (246) and by Winnick, Friedberg and Greenberg (652, 248), who used alanine and glycine in experiments of a similar type. These investigations have been reviewed by Borsook and coworkers (97a) who have also done a very considerable amount of work involving the measurement of the uptake of lysine, glycine and leucine by liver, diaphragm and bone marrow cells, and also by various homogenates and other broken cell preparations. It has been shown that the uptake is of the order of magnitude which might be anticipated from the results obtained with labeled amino acids *in vivo* (section IX, 4). Thus in the experiments of Melchior and Tarver the uptake by liver slices was approximately 0.1–0.2 mg. of methionine per gram of protein per hour, or about 0.5% of the slice protein-methionine was replaced per hour.⁹ With other amino acids uptakes of the same order have been obtained, e.g., alanine 0.05 mg. of alanine per gram of protein per hour (665a).

The uptake by intestinal mucosa (plus muscle) is higher than that of liver slices, and of liver is higher than that of diaphragm

⁹ Borsook and coworkers (97a) made an incorrect recalculation from the data of Tarver and Melchior (436) and arrived at a low figure for the uptake of methionine by liver slices.

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the synthetic process is coupled with the reactions concerned with the release of oxidative energy by the cell. This is also shown by the comparison of the effect of dinitrophenol concentration on phosphate uptake and on alanine uptake which has been made by Lipmann (407a). Since the energy involved in the formation of peptide bonds is of the same order as that concerned in the formation of the glucosidic linkage and since the formation of the latter has been conclusively demonstrated to involve phosphate bond energy, it appears reasonable to anticipate that the formation of peptide bonds will involve reactions coupled in some way with energy rich phosphates.

However, the uptake of lysine by guinea pig liver homogenates does not behave according to these expectations since it is not inhibited by anaerobiosis and is only slightly affected by cyanide and azide. In other respects also the uptake of this amino acid in homogenates behaves in an anomalous fashion; its uptake varies linearly rather than logarithmically with the concentration of the labeled amino acid.¹⁰ It is, therefore, reasonable to suppose that bonds other than ordinary peptide bonds are concerned with the binding of lysine to the homogenate protein, particularly since the amino acid has a second and reactive amino group.

In general the uptake of amino acids by homogenates or the particulate fractions prepared from tissues is lower than that observed with slices, although in some cases this may be obscured by complicating factors. Thus the label may appear in other amino acids (or other reactive molecules) as in serine when glycine is used (652, 653a), and the second labeling agent thus formed is also taken up by the proteins and adds to the total uptake observed. Such side-reactions may occur to an abnormal degree in broken cell fractions.

Melchior and coworkers (435a) have also observed that resting *E. coli* take up methionine, and that here also the uptake is greatly reduced in the presence of the respiratory poisons and by fluoride.

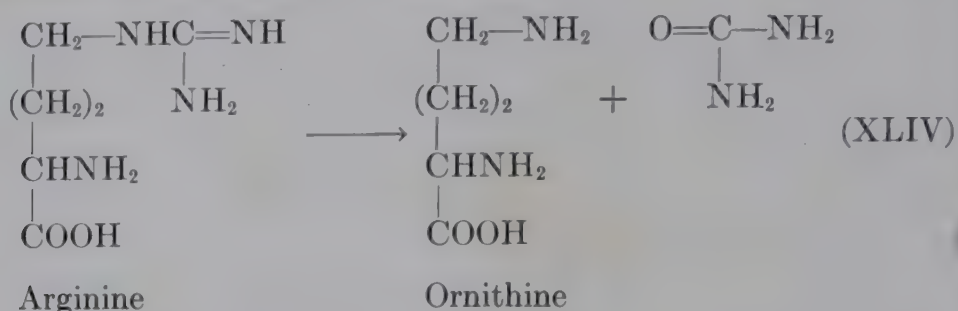
VI. FATE OF THE NITROGEN

1. Urea Formation (494)

Urea is the chief end product of nitrogenous metabolism in mammals, elasmobranch fishes and amphibia, whereas in birds,

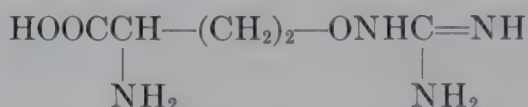
¹⁰ The uptake of methionine shows an apparent deviation from the logarithmic relationship (559a). However, at high concentrations a significant amount of methyl mercaptan may be formed from methionine in slices and homogenates and this mercaptan is bound to the protein as is cystine (cysteine (436)).

reptiles, and insects the end product is uric acid, and in aquatic invertebrates ammonia rather than urea or uric acid. Since the discovery of the enzyme arginase by Kossel and Dakin (354), it has been apparent that at least part of the urinary urea arises by the breakdown of arginine to ornithine.



Arginase is present in the livers of all species producing urea but absent from those producing uric acid. Besides the liver, the enzyme is also present in the spleen and the kidney, in particular in the proximal tubules of this organ (639). It is found in the hepatopancreas of snails (29).

The enzyme shows a high specificity for arginine, although it attacks canavanine,

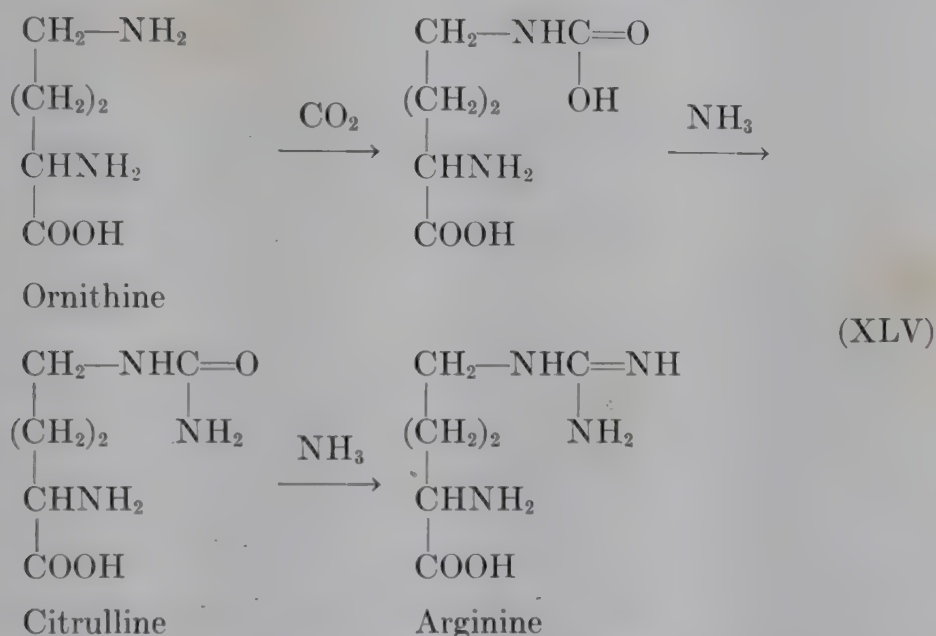


δ -guanidinovaleric acid and α -N-methylarginine. Generally, the enzyme has been reported not to catalyze the hydrolysis of D-arginine, although a preparation from human liver behaved anomalously in this respect (13). Activation of the enzyme occurs with the metallic ions, Mn^{++} , Co^{++} , and Ni^{++} ; inactivation follows treatment with benzoquinone. A dietary deficiency of manganese may lead to a reduction in the arginase activity of the liver (117, 555). The activity of the enzyme is less in the presence of L-amino acids, especially of ornithine (316, 25) but D-amino acids have no similar effect.

A good correlation exists between urea production in different species and the presence of arginase, which suggests that all the urea is formed from arginine. In some way amino acid or ammonia nitrogen must be converted into arginine nitrogen. The correlation is particularly evident when the frog and tadpole are compared (450). The larval form excretes ammonia predominantly and has little arginase in the liver, whereas the adult form excretes urea and has ample arginase in the liver. The change from low to high liver

arginase occurs at metamorphosis coincident with the change in the chief nitrogen excretory product.

A mechanism of urea formation from arginine was proposed by Krebs and Henseleit (375) after extensive investigations with tissue slices. The clue to this mechanism was that ornithine had a large catalytic effect on urea production. Citrulline had a similar effect. In the livers from fasted animals urea production proceeded slowly in the absence of one of these catalysts and glucose, lactate or other easily oxidizable substrate. The cycle may be formulated as follows:



Arginase converts the arginine into ornithine and urea, thus completing the cycle. The actual formation of citrulline was shown by Gornall and Hunter (268), and the rate of urea production evidently depends on the rate of amination of this substance. The cycle received support from work involving mutants of *Neurospora* (575). Apparently, two enzymes are involved, one concerned with the formation of the carbamino compound and the other with its amination.

Although superficially the cycle provides a satisfactory mechanism for urea formation it is not entirely so. It was shown by Borsook and Keighley (113) that *in vitro* the formation of urea is accompanied by an increase in oxygen consumption—urea formation is an endergonic reaction (114). This conclusion is substantiated by the fact that the formation of arginine from citrulline is inhibited by malonate (157, 224) and that the inhibition ceases when fumarate or malate are added (224, 374a), that is, malonate

depresses that fraction of the respiration involving succinic acid as an intermediate. Consequently, the synthesis of urea must be coupled with energy yielding reactions. In other words, although the Krebs-Henseleit cycle may correctly represent the essential reactions concerned with urea formation, yet it lacks the mechanisms necessary to force the reaction to proceed in the direction written. To obviate this difficulty various investigators have endeavored to find ammonia carriers.

The conversion of citrulline to arginine. Leuthardt (392) observed a stimulation of urea synthesis by glutamine and proposed other

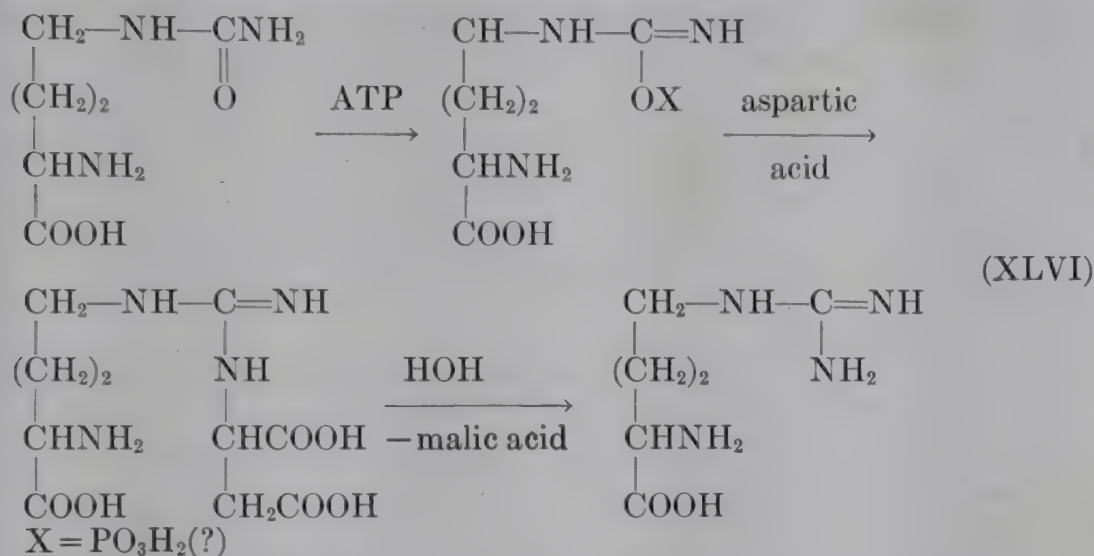
TABLE X
UREA FORMATION IN GUINEA PIG LIVER SLICES

Substrate	Q _{urea}
None	1.26
0.01 M citrulline	1.85
0.01 M glutamate	1.65
0.01 M citrulline; 0.01 M glutamate	2.85
0.01 M NH ₄ Cl	1.79
0.01 M citrulline; +0.01 M NH ₄ Cl	1.91
0.01 M glutamate; +0.01 M NH ₄ Cl	4.48
0.01 M glutamate; +0.01 M citrulline; +0.01 M NH ₄ Cl	7.16

Animals starved 24 hrs. before experiment.
Krebs, H. A. (373).

mechanisms of urea formation in which ornithine was not involved and Bach (23, 26) also believed that more than one pathway of urea synthesis existed as the result of effects he observed with glutamic and α -ketoglutaric acids, but it remained for Borsook and Dubnoff (102) to show that the conversion of citrulline to arginine in guinea pig kidney slices, maintained aerobically, is facilitated by glutamic and aspartic acids. These experiments were confirmed by Krebs (373) working with slices from both livers and kidneys of rats and guinea pigs (Table X). Likewise in liver homogenates glutamic acid stimulates arginine formation when adenosine triphosphate and Mg ions are also present (157, 224). Ratner (494, 495a) has shown that glutamic acid has a dual function in this system. It serves both as a substrate the oxidation of which maintains the adenosine triphosphate concentration, and also as a source of aspartic acid which appears to be the specific nitrogen donor. Aspartic acid is less readily metabolized than is glutamic acid in homogenates so that its function as the donor is not apparent unless other more readily oxidizable substrates are added or unless

a system generating adenosine triphosphate anaerobically is present. The function of the adenosine triphosphate appears to be to form an active citrulline derivative which is capable of spontaneously condensing with aspartic acid to form an intermediate which on hydrolysis gives rise to malic acid:



The intermediate has been isolated in impure form. It has also been shown that two different enzymes are involved, one concerned with the adenosine triphosphate reaction and the other with the hydrolytic decomposition of the intermediate. The specificity of the aspartic acid is shown by the effect of malonate on the system. In homogenates glutamate gives little arginine in the presence of this inhibitor because its oxidation cannot proceed beyond the succinate stage; no oxalacetate is formed and hence aspartate cannot be produced (by transamination). It should be remarked that the liver homogenates used by Ratner and Pappas were rich in arginase, so the formation of arginine was assumed from the appearance of the urea—the product actually determined.

If reaction XLVI is examined it will be seen that with respect to aspartic acid it amounts to a hydrolytic deamination. It remains for the future to determine if the specificity of this reaction is such that only this amino acid can be so deaminated. At any rate the existence of this type of deamination makes it possible that glutamic acid and alanine, as well as aspartic acid, lose their amino groups to form urea directly, without any intermediary formation of ammonia (see ref. 393).

Krebs and coworkers (374b) have subjected the Ratner-Pappas mechanism of arginine formation to some criticism which, however, is not of sufficient moment to discredit the theory.

The conversion of ornithine to citrulline. Borsook and Dubnoff (107) have shown that when liver homogenates are supplemented with glutamate, oxalacetate, ammonia, and adenosine triphosphate, it is possible to get a formation of urea from ornithine. In the absence of adenosine triphosphate a substance with the properties of citrulline accumulates. The enzyme system has been further investigated by Cohen and Hayano (158, 159) who replaced the adenosine triphosphate with adenylic acid and also added Mg ions. They also showed that the enzyme system was contained in the readily sedimentable particles of the homogenate. The carbon for the synthesis apparently comes from carbaminoglutamic acid (156) but as yet the actual mechanism of ammonia transport has not been elucidated.

As might be expected on the basis of arginase distribution, the site of urea formation is the liver. This was shown in perfusion studies by Schroeder (530) and by Bollmann, Mann and Magath (92, 420) in the hepatectomized dog which neither deaminates amino acids nor forms urea. Consequently, in conditions in which considerable liver damage exists urea formation may be reduced (576).

Fed urea is apparently only converted to a small extent to ammonia in the rat according to the experiments of Bloch using urea- N^{15} . However, were ammonia formed in the mammal, it would be reconverted to urea with great rapidity in the liver (574). Consequently the small amount of N^{15} in the ammonia and amino acids in the Bloch experiments may give a false impression of a small conversion, whereas the true conversion may be much larger. In the rabbit injection of urea may result in the formation of toxic amounts of ammonia (32, 35).

More recently it has been shown, using urea labeled with C^{14} , that in the mouse following the intraperitoneal injection of this substance, 20% of the C^{14} appears in the respiratory carbon dioxide in 24 hours (386a). Approximately all the rest of the urea carbon appears in the urine in the same form as that injected. The mechanism whereby urea is decomposed is not known but certainly these experiments should be repeated in different species and particularly in animals with the gastro-intestinal tract removed in order to completely exclude bacterial action within the canal.

Urea is apparently quite freely diffusible throughout the body water. Its concentration in the blood shows some fluctuation from the normal level of about 12 mg.% in the human subject, corresponding to the level of protein catabolism. After a protein

meal its maximum rate of excretion is reached after five to seven hours but variable amounts of urea may be retained by the organism since its rate of excretion depends also on the simultaneous loss of water. Thus, the rate may be increased by diuresis. In the nephrectomized animal it is possible to use changes in the urea concentration in the blood as an index of the amount of protein nitrogen catabolized in any given period of time (186a, 217a).

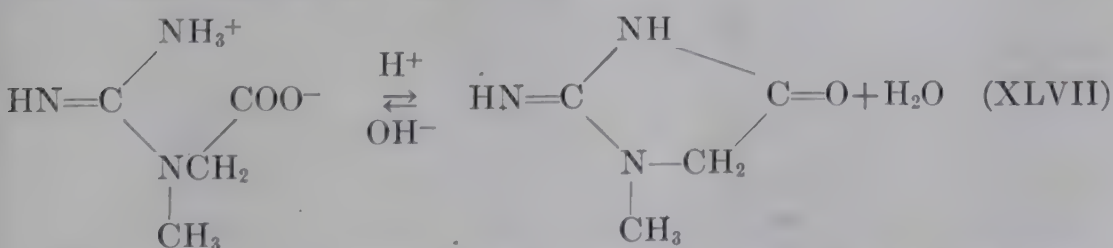
The diurnal fluctuations in rate of total nitrogen output are seen in Fig. 1 taken from the data of Sprinson and Rittenberg (574a). Since urea makes up the greater part of the total nitrogen similar fluctuations would have been found if the urea had been measured.

2. Creatine-Creatinine Formation (620)

Although creatinine, the anhydride of creatine, is one of the relatively minor constituents of the urine, being excreted at the rate of 1-2 gm. per day, its importance should not be minimized. The most remarkable thing about this component of the urine is its constancy in spite of wide variations in the protein component of the diet. This constancy is shown in the creatinine coefficient which is the milligrams of creatinine excreted per kilogram of body weight per twenty-four hours. In the male, it lies between 18 and 32; in the female, between 9 and 26. Children show lower values for the coefficient and in the adult its precise value is determined by the muscular development.

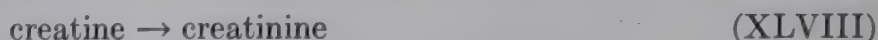
Creatine is not found in the urine of adult normal males, although it may be present in the urine of the female and is quite generally found in infants' urine, but in starvation and in certain pathological conditions it occurs in adult male urine. It is present also in the excreta of birds (480).

In the test tube creatine is readily converted to creatinine and vice versa, depending on the pH.



However, the velocity of the forward reaction *in vitro* is not sufficient to account for the rate of creatinine excretion observed *in vivo* (454). The rate of creatinine elimination is proportional to

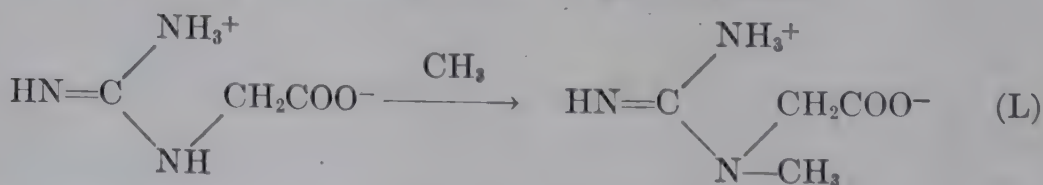
the body creatine (105, 453). Much of this body creatine is in the form of phosphocreatine (phosphagen), which is spontaneously converted into both creatinine and creatine. Thus, the rate of creatinine formation in the organism is the net result of the progress of two non-enzymatic reactions as shown by Borsook and Dubnoff (105):



Fed in small doses, creatine does not appear in the urine as such but is retained by the organism. In larger doses it may be excreted as extra creatinine (512, 45). When labeled with N^{15} the nitrogen of fed creatine is found to be excreted as creatinine with none appearing as urea or ammonia (87). Fed creatinine appears in the urine as such but does not appear as muscle creatine. Therefore, in the body creatine is converted into creatine but the reverse reaction does not occur, at least to any considerable extent.

Many substances have been proposed as precursors of creatine, *e.g.*, arginine, histidine, glycine, choline, and betaine. In general, the feeding of none of these substances gives rise to a substantial increase in urinary creatine or creatinine. Increases are extremely difficult to elicit by dietary means. The evidence regarding creatine formation, therefore, has come from work with isotopes and tissue slices.

Czernecki (177) considered the formation of creatine to be a reaction involving the methylation of glycocyamine:

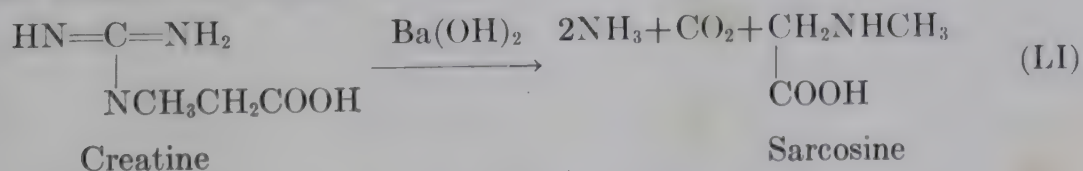


This reaction was shown to take place in liver slices of a large number of species tested by Borsook and Dubnoff (99), but it does not proceed in the kidneys except in the case of the pigeon (100). The methyl group is carried by methionine but not by other amino acids, choline, caffeine, or betaine. However, methylation with choline can take place in the presence of homocystine (103). This transfer of the methyl group from methionine to form creatine (and choline) was shown by du Vigneaud and coworkers (624) in studies in which the methyl group was labeled with deuterium. The labeled methionine was fed to rats for several weeks, then

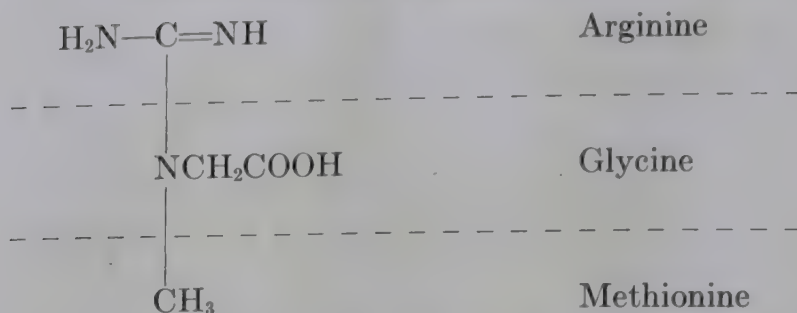
choline and creatine were isolated from the tissues as well as creatinine from the urine. Deuterium was found in the methyl groups of these three substances in the same concentration. Similar transmethylation reactions apparently take place in the human being (560). D-methionine is ineffective as a methylating agent except insofar as it can be changed into the natural form (285). Similarly, labeled choline also gives rise to labeled creatine, but once fixed in creatine or creatinine the methyl group loses its lability (622).

Interest, therefore, centers upon the production of glycocyamine. It was supposed by Knoop (342) that this compound arose by the breakdown of arginine through guanidinobutyric acid. Beta oxidation presumably converted this substance into the corresponding acetic acid derivative. Although later work shows that part of the arginine molecule is utilized for the formation of glycocyamine the rest of the molecule actually comes from glycine. The reaction takes place in kidney tissue (101) from a variety of species. It is referred to as *transamidination*. Transamidination has not been shown to take place in any tissue outside the kidney. In the intact animal, a formation of glycocyamine, which is excreted in part in the urine, occurs following the simultaneous feeding of glycine and arginine (109). Less is formed when the amino acids are taken separately. A similar situation prevails in the fowl where the creatine content of the muscle can be raised by the simultaneous feeding of glycine and arginine but not by feeding of either amino acid alone (295).

The picture was completed by the work of Bloch and Schoenheimer (89) who, by labeling the nitrogen atoms of glycine, arginine (guanidine group), and of guanidinoacetic acid, were able to show that the nitrogen of these compounds is converted to creatine nitrogen. The position of the nitrogen was shown by hydrolysis of the isolated creatine:



The arginine nitrogen gave rise to the guanidine group, the glycine nitrogen to the sarcosine part of the molecule. None of the creatine nitrogen came from urea or choline but sarcosine was almost as good a source of nitrogen as glycine (88). Therefore, the sources of the creatine molecule can be pictured as follows:



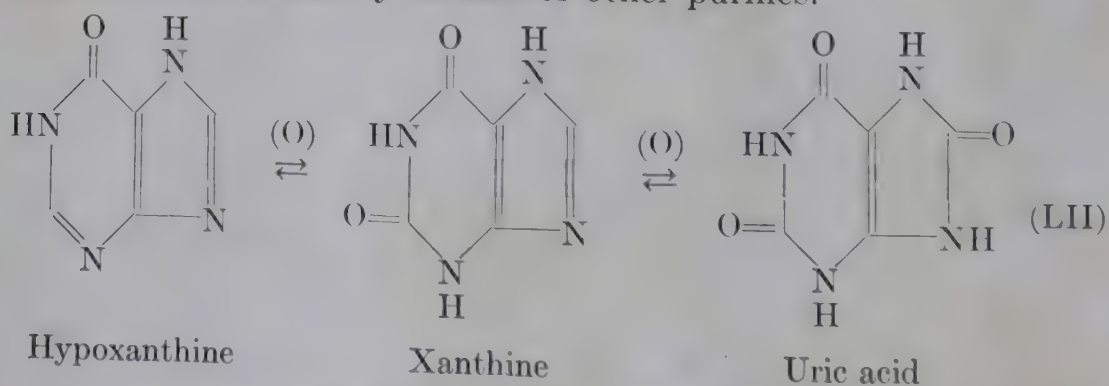
Methylation will be dealt with further in the section on methionine.

The half life of creatine in the body has been determined by labeling the nitrogen with N^{15} (90) and also by labeling the methyl group with deuterium (164). So determined, they were 29 and 36 days respectively, which means that about 2% of the body creatine was replaced per day. The rate of replacement is said to be unaffected by the methionine content of the diet (164).

3. Uric Acid Formation

Uric acid occurs as the major end product of nitrogen metabolism in birds, reptiles and insects. In mammals it comprises a variable percent of the excreted nitrogen depending on the nature of the diet, because it can arise indirectly by synthesis from ammonia nitrogen or directly from dietary or endogenous purine. The formation of uric acid from the immediate purine precursors has been extensively studied. These precursors, of course, occur in and can be formed by the breakdown of endogenous or exogenous nucleic acid.

The enzyme, xanthine oxidase, catalyzes the oxidation of hypoxanthine and xanthine to uric acid (269), and also catalyzes the oxidation of aldehydes and of other purines.



It is present in the liver and kidney of animals, with more present in the former tissue. The enzyme is a flavoprotein (30) with isoalloxazine adenine dinucleotide as prosthetic group. The action of the enzyme results in the formation of hydrogen peroxide when

oxygen acts as hydrogen acceptor, but oxidations with the enzyme are also possible in the presence of methylene blue.

The conversion of various purines to uric acid by rat tissues has been followed by Borsook and Jeffries (111). The most active tissues were found to be liver, intestinal mucosa and kidney in the order named.

The mechanism of uric acid formation from ammonia has been investigated by Schuler (531), by Krebs (46, 209, 474), and their coworkers. In the pigeon, uric acid is formed in neither the liver nor the kidney alone, but from both together. The liver forms a stable precursor, which was shown by Krebs to be hypoxanthine.

TABLE XI
HYPOXANTHINE FORMATION IN PIGEON LIVER SLICES

Substrate Added	Q _{hypoxanthine}
None	0.90
0.02 <i>M</i> glutamine	1.95
0.02 <i>M</i> glutamic acid	0.99
0.0033 <i>M</i> NH ₃ +0.02 <i>M</i> oxalacetate	1.97
0.02 <i>M</i> glutamine+0.02 <i>M</i> oxalacetate	2.54
0.0033 <i>M</i> NH ₃ +0.02 <i>M</i> pyruvate	1.72

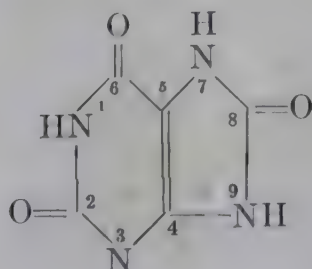
Örstrom, A, Örstrom, M, and Krebs, H. A. (474).

The hypoxanthine is converted to uric acid by kidney tissue. Apparently, liver is deficient in xanthine oxidase in this species. In the hen, both reactions occur in the liver. For these reactions to proceed it is necessary that the cell structure be intact.

Concerning the precursors of the uric acid carbon, there is little information from these experiments. Contrary to older reports, mesoxalic acid and tartronic acid (hydroxymalonic acid) are without influence on uric acid formation. The formation of uric acid is stimulated by oxalacetate and glutamine as shown in Table XI.

Uric acid formation is not stimulated by urea.

The origin of the carbon and N atoms of uric has in part been elucidated by feeding glycine labeled with isotopic N (550, 551), and CO₂, lactate, formic acid, acetate, and glycine labeled with C¹³ (570, 132).



N_7 and probably C_4 and C_5 come from glycine.

C_6 comes from CO_2 .

C_2 and C_3 come from formic acid.

From these results and those previously cited, it appears that glutamine serves as a donor of the nitrogen in positions 1 and 9. Moreover, the effect of oxalacetate must be that of non-specific adjuvant for the system.

If the sole origin of N_7 is glycine nitrogen, then the bird, in order to excrete three moles of nitrogen, must be provided with one mole of glycine. This perhaps explains the glycine requirement in the fowl, if glycine is not formed from serine.

It should be emphasized again at this point that uric acid is essentially an end product of metabolism. The demonstration of the introduction of various isotopes into the molecule presumably indicates that purines were synthesized from precursors containing the labeled atoms and that thereafter these substances were catabolized to form uric acid. Since it does not appear to be entirely pertinent to go into all the recent investigations concerning purine and pyrimidine metabolism it will suffice to mention that Barnes and Schoenheimer (34, 131) showed that rats and pigeons fed isotopic ammonia converted it into purine and uric acid. Later, Plentl and Schoenheimer (484) showed that in the rat fed guanine, the N is excreted as allantoin, in the pigeon as uric acid. Uracil and thymine nitrogen (pyrimidine nitrogen) on the other hand, were excreted as urea and ammonia nitrogen.

VII. OPERATION IN THE WHOLE ANIMAL OF ENZYME SYSTEMS INVOLVING NITROGEN

It has been seen from the preceding sections that there are in the tissues enzyme systems capable of removing, transferring and adding nitrogen from and to carbon chains as well as for the disposal of the nitrogen. The net results of the operation of these enzyme systems in the whole animal can be seen in various ways.

They are evident in experiments of a nutritional type discussed in the preceding chapter. Thus, when a D-amino acid, corresponding to a lacking essential amino acid, is fed there is often a substitution by the D-form for the L-form. Presumably the optical inversion which must occur involves first a deamination followed by reamination of the keto acid to give the natural form. When deamination cannot occur, as in the case of lysine, then the D-form does not replace the missing L-amino acid. Apparently the reverse

is not invariably true because many D-amino acids which are more or less readily deaminated by the oxidase, *e.g.*, isoleucine and valine, fail to substitute for the missing L-forms. The reason for this failure is not apparent. It can hardly be due to the fact that the keto acid is being catabolized too rapidly, or that it is not being reaminated at a sufficient rate because, when fed, the appropriate keto acids do substitute for isoleucine and valine. It may be that the D-amino acid exerts an inhibiting effect on the reamination or on the incorporation of L-amino acid into protein.

As might have been expected from the results obtained *in vitro* some of the essential amino acids methylated on the nitrogen are utilized for growth. These results are explicable on the same basis as those just discussed. It would be of interest to know whether N-methylleucine, isoleucine and valine substitute for the corresponding essential amino acids because of the apparently anomalous behavior of the keto acids. Where deamination is impossible, due to the absence of hydrogen on the α -carbon, catabolism of the amino acid is inhibited (387).

The operation of the systems concerned with the metabolism of nitrogen is again, and most strikingly shown by the results of Schoenheimer and others using amino acids labeled with N^{15} in the α -amino group. Experiments have been done with glycine (496, 547), the L and D-leucines (526, 497), DL-tyrosine (525), the L and D-lysines (643, 672), and L-aspartic acid (662). When these labeled amino acids were fed to rats over a three day period (10 days in the tyrosine and 4 days in the lysine experiments) in amounts about double those present in the dietary protein, it was found on sacrificing the animals that a large part of the nitrogen had been incorporated into the tissue proteins. However, the N^{15} in the proteins was not confined to the amino group of the amino acid fed but was also found in the α -position of all the amino acids examined with the exception of lysine and of threonine (610a). When the labeled amino acid was of the natural configuration, the highest isotope concentration was always found in this amino acid but with the aspartic acid acting as an exception. In this case the highest N^{15} concentration was found in the glutamic acid. This is in agreement with the enzyme studies which show that the most rapid transaminase reaction is that between glutamate and oxalacetate (reaction XVIII). The next highest isotope concentration, with one exception, always occurred in the glutamic acid with somewhat less in the aspartic acid. Still less was found in other

amino acids. This is in agreement with the observations which show that glutamic acid is the only amino acid readily synthesized *in vitro*. The high isotope content in the aspartic acid could be accounted for either by direct synthesis or by transamination. In this connection it would be of interest to know the N^{15} concentration in alanine—the other amino acid definitely concerned in transamination. How nitrogen enters into the formation of the other amino acids is not clearly shown by any *in vitro* experiments but the appearance of the label clearly shows the existence of some mechanism of nitrogen incorporation. Moreover, after feeding D-leucine it was observed that N^{15} was present in higher concentration in the dicarboxylic acids than in the L-leucine isolated from the proteins. Optical inversion must have taken place as a result of complete deamination followed by reamination.

The process of reamination must be a very rapid one as shown by a comparison of the amount of N^{15} which was taken up from the dietary L-leucine by the proteins in the form of leucine with that taken up in the form of other amino acids. A total of 57.5% was incorporated into the proteins of the animals. Of this amount, it was calculated that 17.8% was actually leucine N^{15} with the balance (39.7%) in amino acids other than leucine. Thus, a very large part of the L-leucine fed must have suffered loss of N^{15} during the time it remained as free amino acid and much of this nitrogen was not excreted but became reattached to carbon to give new amino acids. In addition, when D-leucine was fed, the total incorporation of N^{15} into the proteins was 34.4%, not very different from the total incorporation when the L-amino acid was fed, and quite similar to the incorporation as amino acids other than leucine (39.7%). The amount of nitrogen undergoing transfer was therefore closely similar whether D or L-leucine was fed. It would be of interest to know if this result is due to coincidence or to the fact that the rate of reamination is the rate determining step in the process, or whether in the case of the L-amino acid some transaminating type of mechanism prevails.

These experiments also showed that, in part at least, D-leucine provided the carbon chain for the formation of L-leucine. The D-leucine besides being labeled with N^{15} also contained stably bound deuterium which made it possible to follow the fate of the carbon chain. The ratio in which these two isotopes occurred in the dietary amino acid was determined (D/N ratio). After feeding, the ratio was redetermined in leucine isolated from the proteins.

TABLE XII

DEUTERIUM AND N¹⁵ IN L-LEUCINE, L-LYSINE, OR L-PROLINE OF
PROTEINS AFTER FEEDING THE DOUBLY LABELED AMINO ACID

	D	N	D/N
<i>Labeled L-Leucine fed (ref. 526)</i>			
Liver Leucine	24.4	15.8	1.54
Carcass Leucine	6.7	3.8	1.76
<i>Labeled D-Leucine fed (ref. 497)</i>			
Liver Leucine	9.8	0.8	12.3
Carcass Leucine	1.5	0.3	5.0
<i>Labeled L-Lysine* fed (ref. 643)</i>			
Total Protein Lysine			
Expt. I	41.7	41.4	1.01
Expt. II	40.2	38.7	1.04
<i>Labeled L-Proline† fed (ref. 587)</i>			
Organ Protein Proline	45.5	40.7	1.12
Carcass Proline	8.8	8.8	1.00

* Total dietary lysine recalculated from Snell, E., Ann. N.Y. Acad. Sci., 47, 161 (1946) and Horn M. J., Jones, D. B., and Blum, A. E., J. Biol. Chem., 169, 71 (1947). At. % N¹⁵ excess = 0.71, at. % D = 2.59.

† Total dietary proline recalculated from data of Brand, E., Ann. N.Y. Acad. Sci., 47, 187 (1946). At. % N¹⁵ excess = 0.89, at. % D = 6.8.

Results calculated to a basis of 100 At. % D & N excess in the particular optical form of the total dietary amino acid i.e., D/N = 1.

A small increase was found following the feeding of L-leucine, a large increase after D-leucine, as shown in Table XII. So there must have been a loss of N from the carbon chain followed by its reamination. The loss of N from the carbon chain is not followed necessarily by its immediate catabolism.

When L-lysine labeled with deuterium was used, the D/N ratio in the protein lysine was not changed showing that lysine had suffered no loss of nitrogen. This is in accord with the other results which showed that lysine did not exchange nitrogen. With respect to the behavior of its α -amino nitrogen, lysine is evidently in a class by itself, although according to the preliminary results of Thompson and Tickner (610a) threonine is in the same class.

These experiments show that a continual shifting of nitrogen between the free amino acids of the tissues occurs in animals. This is doubtless of great importance in the animal economy and may result in the conservation of the essential basic (carbon) structures of amino acids in short supply by a continual reamination with nitrogen previously associated with amino acid in less demand.

The experiments so far cited do not show whether free ammonia is involved in the nitrogen shifts. That free ammonia does react may be deduced from the utilization of nitrogen following D-leucine feeding, because transaminase is not concerned in the deamination

of the D-acid. Consequently, either deamination to give ammonia occurred or else deamination took place by some undiscovered mechanism. However, the utilization of ammonia for amino acid synthesis has been shown directly by experiments in which ammonium salts containing N^{15} were fed (244, 507). The nitrogen which appeared in the protein was in highest concentration in the amino groups of the glutamic acid, next in the aspartic acid, with less in the other amino acids isolated and with virtually none in the lysine. These results are obviously in accord with those previously given. It should be mentioned that when L-glutamic acid labeled with N^{15} was fed, the distribution of the nitrogen in the proteins was much the same as after giving ammonia (505). The amino acid must have been deaminated or transaminated extremely rapidly.

More recently Sprinson and Rittenberg (574a) have reported important experimental work which involved the feeding of N^{15} labeled ammonium citrate to rats and human subjects maintained on diets of different protein content. The rate of excretion of the isotope in the urinary ammonia, urea and total nitrogen was followed. One of the most interesting findings, pertinent to the present discussion, was that the isotope concentration in the urinary urea always remained greater than in the ammonia.¹¹ In previous work with N^{15} labeled amino acids, *e.g.* glycine (496, 547), it had been found that the relationship between these two concentrations was the reverse; the isotope concentration in the ammonia was higher than in the urea. An exception was provided by L-aspartic acid (662a). When D-amino acids (497, 644) or DL-amino acids (525, 586) were fed the same results as with glycine were obtained. The indication is that the nitrogen of aspartic acid and also that of ammonia is more nearly related to that of urea than is the nitrogen of either the L or D-amino acids. The observations with aspartic acid are in accord with the enzymic investigations of Ratner, which, as previously noted in Section VI, 1, show that this amino acid is the specific nitrogen donor for the conversion of citrulline to arginine in the urea cycle. Ammonia leads to the same results because of its rapid conversion to urea in the liver via the intermediates glutamic and aspartic acid. The reason why the other amino acids do not lead to the synthesis of urea with a relatively high isotope concentration probably results from the dilu-

¹¹ Immediately after feeding the ammonium citrate in some experiments the label was in higher concentration in the urinary ammonia than in the urea. Presumably in these cases part of the ammonia escaped being trapped by the liver and was excreted with little dilution.

tion of the isotopic ammonia by nitrogen arising from the deamination of unlabeled amino acids and also due to the direct conversion of amino acid nitrogen to urinary ammonia by the kidney.

Sprinson and Rittenberg (574a) also observed that the percent of the labeled nitrogen which was excreted in any given period following the ingestion of a dose of ammonium citrate was a function of the protein content of the diet in both rats and human subjects. On a high protein diet the rate of loss of isotope was high; on a low protein diet it was correspondingly low. The authors interpreted these results as being due, in part at least, to the rapid conversion of the $N^{15}H_3$ to urea. The urea is unavailable for synthetic reactions (compare Section VI, 1) so that the N^{15} so dealt with could no longer be incorporated into amino acids and proteins. However, this is only a partial explanation of the phenomena. On a high protein diet the amount of unlabeled nitrogen and hence ammonia entering the pool per unit time is high so that any isotopic ammonia will suffer an immediate dilution. Since much of this nitrogen is in excess of the anabolic requirements of the animal it is converted to urea and lost in the urine; that is on a high protein diet the exogenous metabolism is high.

If the factors governing the rate of loss of urea from the animal organism are examined the following picture develops. Urea is freely diffusible throughout the body water, where it exists in approximately equal concentration in all compartments. So if the urea is all labeled at a given time then this labeled urea will be *washed out* of the system at a rate depending on the rate of formation (and excretion) of unlabeled urea and on the total amount of urea in the system. The rate of washout in the experiments under consideration may be calculated¹² approximately and it turns out

¹² As a first approximation the washout phenomenon may be treated as a monomolecular reaction and if specific figures from the paper of Sprinson and Rittenberg are employed the time for the concentration of the isotope in the urea in the subject to reach half its original value may be calculated as follows:

From Table II, Subject D. R. 64.4 kilos

$$\text{Body water} = 64.4 \times \frac{70}{100} = 45.1 \text{ kilos}$$

Assuming 12 mg. urea-N per 100 ml.

Then total urea-N = $45.1 \times 0.12 = 5.41$ gm. urea-N.

Average excretion of total N during the experiment = 0.553 gm. per hour.

Assuming 85% urea-N in total urine N.

$$\text{The average excretion of urea-N} = 0.553 \times \frac{85}{100} = 0.47 \text{ gm. per hour.}$$

Applying this data in the monomolecular reaction equation, then

$$\log \frac{a}{a-x} = kt = \log \frac{5.41}{5.41 - 0.47}$$

$$t = 1 \text{ so } k = 0.0395$$

Thus the time for half the urea to be washed out of the system is,

$$t_{\frac{1}{2}} = \frac{0.301}{0.0395} = 7.6 \text{ hours}$$

(continued on following page)

that the urea- N^{15} concentration should reach half its initial value in about 8 hours, where the value is overestimated rather than underestimated. Experimentally it is observed that the excretion rate follows the monomolecular law (Fig. 1) with a half-time of 38 hours in one subject. Moreover, the same half-time for urea- N^{15} is found if, instead of ammonia, N^{15} labeled glycine is fed and if the figures for total nitrogen are used (Fig. 1) (574). So the rate of loss of isotope into the urine proceeds more slowly than would be predicted on the assumption that a washout mechanism only is concerned.

It, therefore, becomes necessary to postulate some mechanism to account for the slowing down of the process of isotope loss. Sprinson and Rittenberg (574) assumed that the mechanism involved was one of protein synthesis and proceeded to derive from a mathematical treatment of the data various figures purporting to show the size of the nitrogen pool and the rate of protein synthesis in the subjects investigated. In order to do this it was necessary for the authors to make various assumptions, the most important of which was that there exists one homogeneous metabolic pool of nitrogen compounds which are used to provide nitrogen for the synthesis of body protein. The constituents of this pool are presumably in the NPN fraction and comprise such substances as amino acids, amino acid amides and ammonia, which may come into the pool either from the diet or from endogenous sources. Since it is well known, for instance, that the concentrations of amino acids in different tissues are highly variable, it is hazardous to start out with such an assumption. Moreover the data of Shemin

(continued from p. 829)

It is true that this calculation is made on the assumption that the labeled urea is equal in concentration throughout the body compartments and that the urea formed has the same washout effect on all compartments. Since both the isotopic urea and the washout urea are formed mainly in the liver these assumptions have a limited validity, and will lead to an overestimation of the half-time of urea in the subject. Thus if all the isotopic ammonia fed or any given fraction thereof, were immediately converted to urea the isotope concentration in the total body urea should fall to half its original value in about 8 hours. Since the urinary urea isotope concentration, in this case, may be taken to be a fairly close reflection of the total body urea isotope concentration then the urinary urea- N^{15} concentration should fall to half its original value in this 8 hour period.

When the experimental data (Subj. D.R., Table II) are plotted it is found that the half-time is 37 hours rather than eight. Similarly, after glycine feeding, using the data of Table IV and subject DS-1, the time turns out to be about 38 hours. In this experiment and others, as might be expected, the change in isotope concentration in the total nitrogen behaves very similarly to that in the urea, since the urea comprises the major part of the total. When the glycine data in experiments DR-1, and DR-2 from the following paper of Sprinson and Rittenberg (574) is used the times required for the isotope concentration in the total nitrogen to reach half its original level turn out to be 37 and 32 hours respectively. Consequently whether ammonia or glycine is used, the half-time of the urea (or total nitrogen) in the subjects, judging from the urinary data, turn out to be very similar.

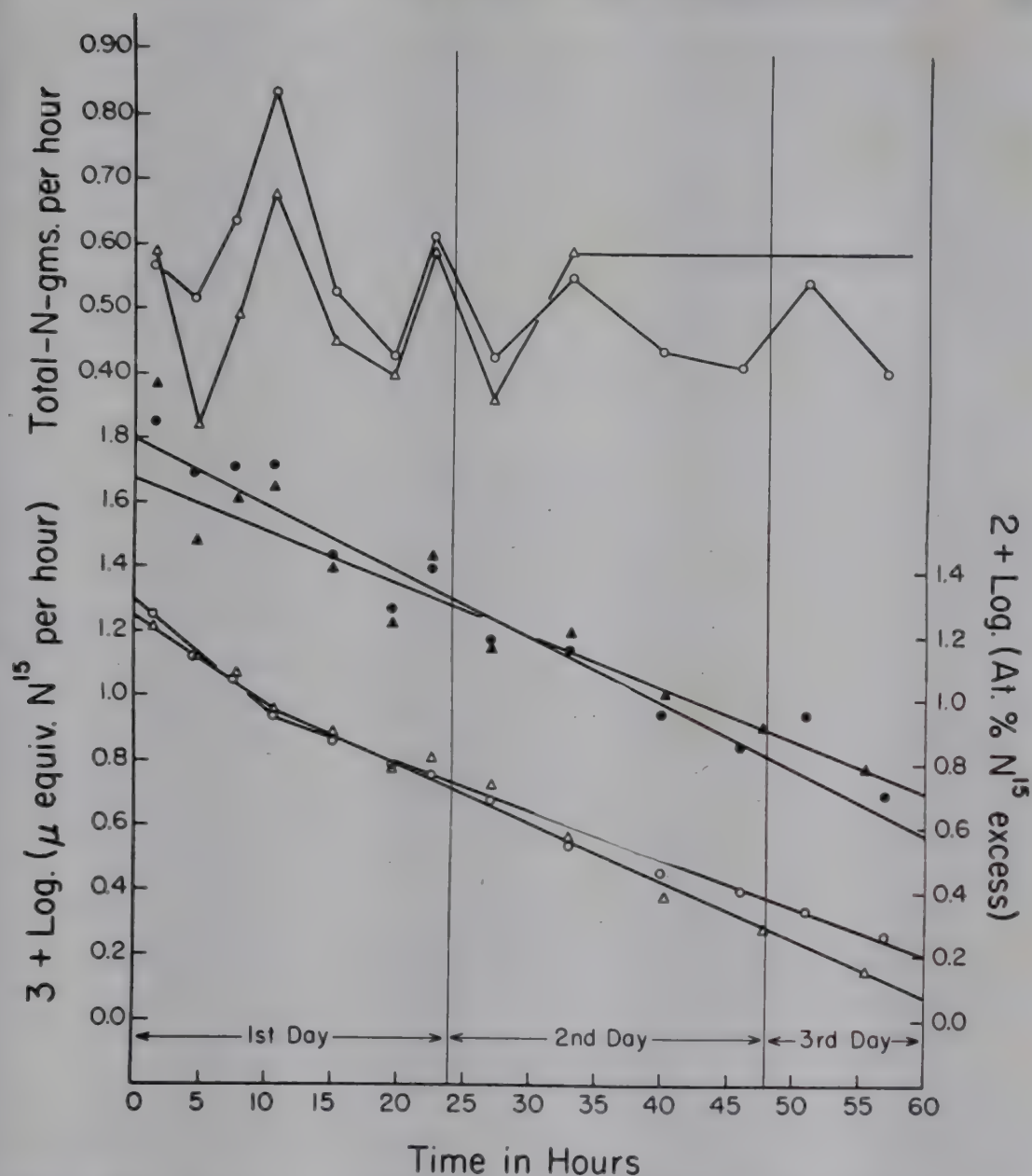


FIG. 1. The rate of excretion of total nitrogen and N^{15} by a human subject during a 60 hour period following the ingestion of glycine- N^{15} . Upper pair of curves, total nitrogen excretion; middle pair, log. of N^{15} concentration in total nitrogen excreted. Data from Sprinson and Rittenberg (574); subject D.R., expt. 1 triangles, expt. 2 circles.

and Rittenberg (547) show that with respect to N^{15} concentrations there is no homogeneity in the NPN fractions from various tissues. There is, in fact, a great and striking, inhomogeneity. Also, from the preceding discussion of the washout phenomenon with respect to urea, it is seen that there is another type of nitrogen pool within the animal which includes, besides the compounds in the metabolic (anabolic) pool, also catabolic end products such as urea, uric acid, and creatinine (catabolic pool), in which the labeled nitrogen may appear. Once in this pool it will take a definite time for the

labeled compound to be washed out into the urine. It is, therefore, questionable whether there is any justification for the mathematical treatment to which Sprinson and Rittenberg submit their data.

It should also be remarked that Sprinson and Rittenberg find an average pool size of 36 gm. for a 70 kg. individual. If the pool largely comprises free amino acids this appears to be rather large. It might also be anticipated that the amount of nitrogen entering the synthetic system would be a function of the quantity of protein in the diet. The estimated values do not show any such correlation; in fact rather the reverse.

In experiments with N^{15} in which the distribution of N in the arginine molecule was investigated, little of the isotope was found in the ornithine part, most being present in the amidine group (244, 547, 587). The lability of the nitrogen in this group is thus demonstrated. It necessarily has to be labile if arginine is a source of urea. In the experiments of Shemin and Rittenberg (547), with glycine labeled with N^{15} in the diet, it was found that, at the time when liver protein arginine could be assumed to have attained the same isotope concentration in its amidine group as the free liver arginine, the urinary urea showed a similar concentration of N^{15} . This supports the position of arginine as a source of urea N but does not exclude the dicarboxylic acids and their amides as sources. No matter what the form of N^{15} fed, relatively high concentrations of N^{15} were found in the amide N of the proteins.

VIII. FATE OF THE CARBON RESIDUE

Superficially, the feeding of amino acid or protein results in the oxidation of most of the carbon and hydrogen to carbon dioxide and water, so that these substances ordinarily supply a considerable fraction of the total caloric requirement. A smaller part may be stored as body protein. However, under appropriate experimental conditions it is possible to show that some amino acids cause a net increase in carbohydrate or acetate or acetoacetate.

1. The Formation of Carbohydrate

The amino acids which are apparently converted into carbohydrate are called glycogenic (glucogenic) amino acids. It is reasonable to anticipate the existence of such a metabolic pathway in the case of some amino acids because the keto acids which result from their deamination are identical with the products of the intermediary metabolism of carbohydrate which can be reconverted to the parent substance. Such keto acids are those corresponding

to the amino acids alanine, aspartic acid, and glutamic acid. It is less clear how the carbon of other glycogenic amino acids is converted to carbohydrate.

A wide variety of methods have been used to show this production of carbohydrate from amino acids. These are:

- (1) Determination of the extra glucose excreted in the depancreatized, alloxan treated or phlorizinized animal.
- (2) Determination of the increase in liver glycogen of fasted animals, generally rats.
- (3) Determination of the action of the amino acid on the blood and urinary ketone substances, acetoacetic acid, β -hydroxybutyric acid, and acetone. These substances are assumed to disappear only when carbohydrate is available for combustion.
- (4) Determination of the fate of the labeled amino acid.

It should be noted that none of these methods, save only the last, can provide real proof of the origin of the carbon fragments. The results of studies of types 1-3 are presented in Table XIII.

They may be summarized as follows:

- (1) Amino acids with unbranched chains and containing 2 to 5

TABLE XIII
FORMATION OF CARBOHYDRATE FROM AMINO ACIDS*

	Extra Glucose After Phlorizin	Deposition Liver Glycogen	Reduction of Ketonuria
Alanine	+DL (178)	+L, D (416, 529 649, 137)	+L, DL (649, 137)
Arginine	+L (178)	+L (140)	+L (140)
Aspartic acid	+DL (504, 382)	+L, DL (136)	+L, DL (136)
Cysteine	+L (178)	-L (435)	-L (135)
Cystine		-L (135)	-L (135)
Glycine	+(504)	+(416, 529, 649, 137)	+(649, 137)
Glutamic acid	+L (413)	+L, DL (136, 227)	+L (137)
Histidine	\pm L (178)	+L, -D (227, 500)	+L (500)
Isoleucine	\pm DL (178)	+DL (134)	-DL (134)
Leucine	\pm DL (178)	-DL (134)	-DL (134)
Lysine	\pm L (178)	-DL (140, 544)	-DL (140)
Methionine	+DL (619)	-DL (435)	
Phenylalanine	-DL (178)	+DL (138)	+DL (138)
Proline	+L (178)		
Serine	+DL (178)	+DL (529, 135)	
Threonine		+DL (283)	+DL (283)
Tryptophan	\pm L (178)	-DL (94)	+DL (94)
Tyrosine	-L (504)	\pm L (141)	-DL (141)
Valine	\pm L (178)	+DL (139)	+DL (139)
	+DL (513)		

* References in brackets.

carbon atoms, yield extra carbohydrate (lysine behaves exceptionally).

- (2) Amino acids with branched chains do not give extra carbohydrate.
- (3) Aromatic amino acids, except histidine, do not yield extra carbohydrate.
- (4) Proline gives extra carbohydrate.

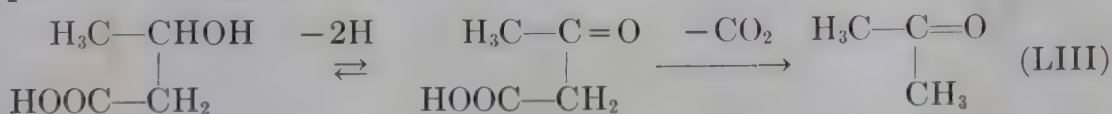
From the table it will be noted where the results lack accord, in particular with regard to the behavior of valine, methionine, and phenylalanine. To this data it may be added that β -alanine is non-glycogenic (169, 529). In many cases the apparent conversion of amino acid to carbohydrate may be referable to a sparing of endogenous carbohydrate or other substance readily converted to carbohydrate by the provision of readily metabolizable substance. The behavior of glycine is particularly interesting in this respect. In short term experiments, it fails entirely to cause deposition of glycogen (498, 651), but in experiments extending over a longer period, it is definitely glycogenic (137, 415). Rats fed glycine are also reported to maintain their liver glycogen following a fast better than similar animals fed alanine or glucose (613). Similarly the same species when pre-fed glycine and then given insulin maintains its liver glycogen better than do untreated animals (613a). Since the amount of extra free glycine in the tissues of the experimental animals is insufficient to account for the difference in glycogen levels the authors assumed that glycine stimulated gluconeogenesis. This was supported when it was shown that the effect was lacking in the adrenalectomized animal (613). Stimulation of gluconeogenesis by glycine was also evoked as an explanation of the low labeling of the liver glycogen in mice fed the amino acid with a carboxyl label (472). Now that the conversion of glycine to serine is known, together with the fact that this conversion is rapid another explanation of these phenomena is obvious. Anomalous results with glycine may also be, in part, due to the slow penetration of this amino acid into cells (172). Not only does glycine penetrate cells slowly but it also appears to interfere with the penetration of other amino acids. Glutamic acid behaves in quite an opposite manner (172, 260).

The behavior of alanine, labeled in the carboxyl group, and fed to phlorizinized animals has been compared to that of α,β -labeled lactate fed to similar animals (281). Lactate is known to be converted to carbohydrate quite readily and in the phlorizinized animal

alanine undergoes an apparent quantitative conversion to carbohydrate. In these experiments, extra glucose equivalent to 60–70% of the alanine appeared in the urine. However, in this urinary glucose there was less than 5% of the isotopic carbon. Most of the excreted glucose did not come from the fed substance, but was of endogenous origin. The behavior of lactate was quite different. In this case, 28% of the fed lactate carbon was found in the urinary glucose, of the remainder, 30% of the carbon went to muscle and liver fat and a considerable fraction must have appeared as respiratory carbon dioxide. The great dilution of the label in the excreted glucose following alanine as compared with that following lactate may have been due to the introduction of alanine into protein and the utilization of endogenous alanine for glucose formation. Alternatively, the low labeling with alanine could be attributed to the slower rate of formation of pyruvate from alanine than from lactate.

2. The Formation of Ketone Substances and Acetate

It has long been known that animals under certain conditions, notably high fat diet, starvation, or lack of insulin, excrete ketone substances into the urine. These substances are three in number, viz., acetoacetic acid, β -hydroxybutyric acid, and acetone. The last mentioned can be considered as the product of spontaneous decarboxylation of the first and the β -hydroxybutyric acid as its product of reduction.



When fed, certain amino acids stimulate the production of these ketone substances as does fat, and this is generally attributed to the direct formation of acetoacetic acid from the chain or ring as it exists in the amino acid molecule. However, the work of Swendseid and coworkers (594) has demonstrated that acetoacetic acid may arise by condensation of acetate. Consequently, if an amino acid increases keto acid formation, then either the amino acid is broken down to give acetoacetate directly, or acetoacetate is formed indirectly from acetate, or the amino acid stimulates the formation of acetoacetate from fat or carbohydrate.

The ability to form acetoacetate or acetate can be tested by any one of the following methods:

- (1) By feeding the amino acid to suitably prepared animals

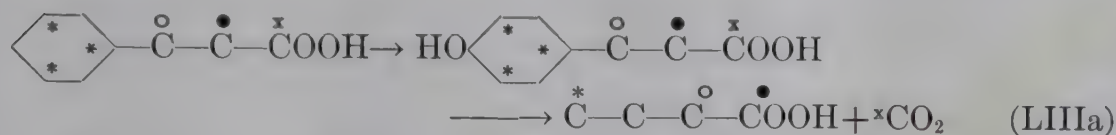
- (animals unable to handle extra keto acid) and determining keto acid in blood and urine.
- (2) By perfusing blood containing the amino acid through the liver and measuring the increase in ketone substances in the perfusate.
 - (3) By incubating the amino acid with suitable preparations, generally liver slices, and determining the keto acid formation.
 - (4) By feeding the amino acid suitably labeled along with an easily acetylated compound, such as phenylaminobutyric acid, and determining the label in the excreted acetyl derivative.

Using these methods it has been shown that leucine, tyrosine and phenylalanine are capable of forming acetoacetic acid. Some doubt is cast on the interpretations of some of the old perfusion studies because ammonium salts were used, and it has since been shown that ammonia increases ketone formation (17, 207) with concomitant decrease in respiration. Ammonium ion is removed rapidly by urea formation in liver slices (and in the whole animal) so that the effect is only apparent with relatively high concentrations.

By the slice method the amino acids mentioned were shown to be ketogenic (208), although the effect of DL-leucine was much greater than of L-leucine so that the ketogenic power may be a property of the D-isomer only (213). The ketogenic nature of tyrosine in slices has been confirmed by other workers (233).

Various derivatives of tyrosine and phenylalanine have also been tested. Embden and Baldes (214) showed that phenylpyruvic acid is not ketogenic, and in slice experiments the compound does not give ketones (208). On the other hand, with both methods (216) *p*-hydroxyphenylpyruvic acid and homogentisic acid give extra acetoacetate, whereas *p*-hydroxyphenyllactic acid gives none (perfusion studies) (463). So the pathway of phenylalanine metabolism according to these results either does not go through phenylpyruvic acid and phenyllactic acid, or only proceeds in part via this pathway. More recently further insight into the mechanism of formation of acetoacetate from phenylalanine and tyrosine has been gained by the use of isotopes. The conversion of the β -carbon of DL-tyrosine to the α -carbon of acetoacetic acid in the intact rat and in liver slices has been shown (653, 642). Similarly the conversion of the α -carbon of phenylalanine to the carboxyl carbon of acetoacetate has been shown to occur in liver slices incubated with DL-

phenylalanine labeled in both the carboxyl group and in the α -carbon (517a). Also the same authors, Schepartz and Gurin showed, using DL-phenylalanine labeled in 1, 3, 5 positions in the ring, that in either tissue slice experiments or in phlorizinized rats the γ -carbon of acetoacetic acid came from positions 1 or 3 of the ring. These results become comprehensible if it is assumed that, during the oxidation of phenylalanine, the side chain suffers a shift in position on the ring (Section X, 9). The data so far mentioned may be depicted as follows:



There is no indication from these experiments that the acetoacetate is formed to any significant extent from two carbon chains by a random condensation, but rather that the four carbon chain preexists in the molecule of the aromatic amino acid. Lerner (391a) has also confirmed these results by using phenylalanine labeled throughout the ring with C^{14} and with C^{13} in the α -position. The C^{14} appeared in the β and γ positions of the acetoacetate and the C^{13} in the carboxyl group as would be expected from what has already been said. When malic acid was isolated from rat liver slice following incubation with the ring labeled phenylalanine, radioactivity was found in the compound, and equally distributed between the carboxyl carbons and the α and β positions. Therefore the four carbons, which remained unaccounted for in the ring, are apparently split out as a unit so that they can form malic acid. When L-tyrosine- β - C^{14} was incubated with liver slices and the malic acid was isolated no C^{14} was found in the acid. Consequently the picture with respect to the mechanism of malic acid formation was confirmed. Thus during the catabolism of the aromatic amino acid four carbons go to form a glycogenic unit and four other carbons go to form ketone bodies, explaining the apparent anomalous results reported in Table XIII.

Bloch (78) fed leucine containing stable deuterium in the molecule along with phenylaminobutyric acid and found deuterium containing acetyl amino acid in the urine. Therefore, L-leucine is converted to acetate. Labeled isovaleric acid was converted to acetate to a comparable degree so that the metabolism of leucine may proceed through this acid. It was previously shown in perfusion studies that the acid gives acetoacetic acid (216). Neither valine nor isobutyric acid form acetate.

IX. THE METABOLISM OF PROTEIN

1. The Partition of Fed Nitrogen and the Concept of Endogenous and Exogenous Metabolism

After an animal has been on a diet containing an adequate amount of protein for a sufficient time, the output of nitrogen in the various channels, of which the urine is by far the most important, becomes equal to the intake. Under these conditions the animal is said to be in nitrogen equilibrium. There may be an output of about 13 gm. of nitrogen in the 24-hour human urine specimen. This nitrogen is ordinarily partitioned about as follows: urea, 86%; creatinine, 5%; uric acid, 1%; ammonia, 3%; with 5% of undetermined nitrogen. Folin (240) examined in detail the changes produced in this partition by increasing or decreasing the total protein in the diet. He found that as the protein content was increased, the percent of the N in the form of urea increased, so that it might, on occasion, amount to 90% or more of the total. The amount of creatinine stayed constant so that the percent nitrogen in this form decreased.

Uric acid was found to behave in a more or less variable manner, but generally showed a significant increase, although it was not usually large—unless the protein fed contained considerable purine. Ammonia also showed an increase which is now known to compensate for the increased acid production associated with a high protein diet. When the protein content of the diet was reduced to a low level, the amount of urea excreted showed a great decrease, so that when the total nitrogen output was about 4 gm., the percent in the form of urea became only 69% of the total. The creatinine output on the other hand, remained about constant so that it showed an increase when expressed as percent of the total. The other constituents behaved as might be expected from what has already been indicated.

Folin was greatly impressed with the constancy of the creatinine output as compared with the variability of the urea, and built a theory of protein metabolism principally on the basis of these findings. The creatinine output was taken to indicate the existence of a constant and low rate of cellular protein catabolism, which he called the *endogenous metabolism*. The fluctuating urea production indicated to Folin the existence of a highly variable protein catabolism. This received the name of *exogenous metabolism*. Thus, the more the total metabolism of protein was reduced by reducing the protein content of the diet, the more prominent became the

endogenous metabolism. The two forms of protein metabolism were thought to co-exist and proceed independently but simultaneously. In most respects the endogenous metabolism corresponds to the "wear and tear" breakdown of protein earlier postulated by Rubner (443).

Creatinine was considered by Folin to be the chief product of the endogenous metabolism, but other substances, such as neutral sulfur and to a lesser extent ethereal sulfates and uric acid, were also assumed to represent products of this form of metabolism. Whether to assign an endogenous origin to any small fraction of the urea and inorganic sulfur was not definitely decided by Folin, although Paton (479) considered some urea to arise from this form of metabolism. It is interesting to note that after 30 days on a protein free diet, the human subject in the experiment of Deuel and coworkers (186) still was excreting urea in considerable amount namely, 50% of a total N excretion of 2.1 gm. At this time, the creatinine represented 27% of the total.

It should be reemphasized that it is extremely hazardous to deduce any general theory of protein metabolism from such data concerning nitrogen partition. It has been seen in a previous section that creatinine is now known to be formed from creatine and its phosphate by a non-enzymatic process; and also that the formation of creatine is concerned directly with the metabolism of three amino acids only, namely, glycine, methionine, and arginine. In addition, the creatine-creatine phosphate content of organisms largely depends on the muscular mass and development. Muscle tissue is not primarily concerned with protein metabolism as is the liver. Therefore, it is improbable that the creatinine output will indicate the level at which any fraction of the protein metabolism is proceeding.

Criticism of the theory appeared, however, for other reasons. The loss of nitrogen and sulfur, which inevitably took place due to the existence of endogenous metabolism, was used to explain the necessity of supplying protein in order to maintain the nitrogen balance. When body protein broke down, there was presumably a complete oxidation which took place in all tissues. Exogenous metabolism, on the other hand, was supposed to be due principally to hydrolytic reactions giving rise to urea and ammonia. Conceived in this way, it was necessary that the endogenous nitrogen requirements be covered only by complete proteins. However, it was found by various investigators that the loss of nitrogen could be partly covered by incomplete proteins (476), incomplete amino acid

mixtures (552), or even by a single amino acid (438), and perhaps ammonium salts (14).

To account for these facts, several hypotheses have been offered: Incomplete protein may inhibit the breakdown of protein in the organism, or protein only breaks down in the organism when its components are required for other purposes, *e.g.*, tyrosine for adrenaline or thyroxine synthesis. From the dynamic point of view it would appear that, in order to prevent net-protein breakdown, a complete mixture of amino acids would be required. If the other alternative is taken, when all amino acids are supplied in sufficient amount, the endogenous metabolism should be reduced to zero. There would be no breakdown of tissue protein at all. This extreme position was taken by Mitchell and coworkers (444), but it is highly improbable that catabolism of body protein can under any conditions be zero. These workers found the non-protein nitrogen (NPN) of rat tissues to remain constant even after 10 days on a diet free of protein. Consequently, they suggested that the endogenous metabolism might be due to the breakdown of this NPN which was derived from tissue protein only when there was insufficient nitrogen in the diet. Endogenous nitrogen only appeared in the urine under conditions of protein starvation.

However, the partial covering of endogenous losses may be explained in another way. When tissue protein breaks down, free amino acids and eventually keto acids are produced. From the previous sections it is evident that if the organism is well supplied with non-specific nitrogen and abundance of calories in a form readily available then the synthetic propensities of the system will be emphasized and the dynamic equilibria will be shifted in such a way as to increase the total protein content of the system. In complete hunger it is well known that the loss of nitrogen into the urine is much greater than when carbohydrate is supplied (414). Also, in other states where there is excessive nitrogen loss, carbohydrate acts in the same way, *e.g.*, in phlorizin poisoning (503, 441). This is usually termed a sparing action of carbohydrate on protein.

Improvement in the utilization of dietary nitrogen by supplying extra calories has been shown by several groups of workers. In dogs, Larson and Chaikoff (384) showed that if extra carbohydrate was fed within a few hours of protein feeding, there was a temporary retention of nitrogen which persisted only so long as the extra carbohydrate was given. Similar results have been obtained in human beings (176).

Thus, the concept of endogenous metabolism is reduced to one which assumes a persistent net destruction of body protein because of oxidation of essential carbon structures and because of the persistent formation of the various nitrogen containing excretory products and their loss into the urine. How this varies with change in the protein content of the diet is not known. In addition, endogenous nitrogen metabolism comprises the processes concerned with turnover of protein which are discussed in section IX, 4 and 5, following the description of experiments showing the incorporation of labeled amino acids into body protein. However, no attempt should be made to draw too strict a line of demarcation between

TABLE XIV

SPECIFIC ENDOGENOUS METABOLISM AND BASAL HEAT PRODUCTION IN DIFFERENT SPECIES

Species	N mg. per kg. Body Weight	Cals. per kg. Body Weight	Ratio N/Cals.
Mouse	614	322	1.94
Rat	213	109	1.99
Guinea pig	178	95	1.91
Rabbit	121	59	2.13
Pig	40.4	19.6	2.10

Smuts, D. B. (563).

the endogenous and exogenous metabolism—between two processes which are so intimately interrelated. The amino acid pool in each tissue is common to both forms of metabolism.

When there is specific nitrogen hunger, the endogenous metabolism is evident in the nitrogen excretion, which after some days on a protein free diet, becomes relatively constant at the specific endogenous nitrogen level. In different species, the specific endogenous nitrogen per kilogram of body weight is quite different (608, 563) and also in the same species at different ages (571, 607, 443), but expressed per calorie of heat produced under basal conditions, it is practically the same in all species (606). Data from Smuts are shown in Table XIV. In addition it has been shown by Mukherjee and Mitchell (448a) that when the basal metabolism of adult male rats is increased by feeding suitable thyroid preparations, then the specific endogenous nitrogen excretion shows a proportionate increase.

These results show that a definite fraction of the basal calories in specific nitrogen hunger comes from the breakdown of body protein. Whether the greater rate of protein breakdown in the

small animal results from the greater demands for calories or whether it is an independent property of the protein metabolizing system cannot be judged from these experiments. Owing to the fact that in the smaller animals there is less muscle with a low turnover rate and content of labile protein, it would appear logical to anticipate a high endogenous breakdown in the small animal.

2. Nitrogen Excretion with Change in Nitrogen Intake

When the protein content of the diet is changed, there is a lag in attaining a new and constant level of N excretion, that is, in regaining nitrogen equilibrium. Thus, when the protein content is reduced from a high level to one lower, for several days or even weeks, there is a positive nitrogen balance, indicating that during this lag period body protein is being broken down. Conversely, fed protein is stored for some days following an increase in the protein content of the diet. Now there are no quantitatively significant differences in the amino acid and peptide contents of tissues at different levels of protein intake; so the nitrogen lag must result from the breakdown or reformation of body protein. Thus, the existence of some "labile" protein in the animal organism is shown.

The kinetics involved in the metabolism of this protein in human subjects were examined by Martin and Robison (423) with very interesting results. They found that the decomposition of this protein proceeded like a first order reaction. Thus, by plotting the log of the difference between the total nitrogen excretion on the days of specific nitrogen hunger (TN) and the specific endogenous nitrogen (EN) against time, a straight line was obtained. Expressed mathematically, the relation was therefore:

$$\log (TN - EN) = a + kt.$$

Other data on nitrogen excretion in specific nitrogen hunger were amenable to the same treatment (429). Seegers (541) followed the excretion of nitrogen by rats fed a nitrogen free diet for a much longer period of time (60 days). When he disregarded the excretion during the first four days he found the following relation to hold:

$$\log TN = a + bt.$$

However, most of this nitrogen does not come from the labile nitrogen.

Borsook and Keighly (115) brought forward indirect evidence to show that this decomposition of labile protein nitrogen still proceeded when the nitrogen balance was maintained. Accordingly,

they gave this persistent breakdown of protein the name *continuing metabolism*. They concluded from their results that on a normal protein diet (10–11 gm. total urinary nitrogen per day) about half of the nitrogen entered the continuing metabolism. Thus, the dietary nitrogen was in large part synthesized into body protein and an equivalent amount of body protein was broken down, so that in any one day the two processes just balanced. Exogenous nitrogen was stored temporarily before excretion, and the extent of storage was thought to be proportional to the nitrogen intake.

This theory of metabolism is obviously quite different from and supplements that of Folin. According to the latter concept, only a relatively small amount of nitrogen enters the endogenous metabolism to replace such nitrogen as is broken down by wear and tear. The theory of continuing metabolism takes into account the labile nature of the body protein. This lability has been shown by two methods, first by the effect of fasting on the protein content of the animal and its organs, and second by the use of isotopes.

3. The Lability of Body Protein (357)

It has been known for many years that the protein content of the organism is variable. However, the increase in the protein content of the organs or tissues of an animal does not generally represent so much an increase in percent protein per unit of tissue—that is a storage of protein in the tissue—as in an increase in the total protein in the whole tissue. There is, as a rule, an increase in size of the organ or tissue with less or no change in percent protein per unit weight. Increase in protein represents an increase in functional protoplasm rather than storage of more or less inert reserve, and is quite different in this respect from the behavior of glycogen and fat. There is no storage of any particular type of protein, at least in the liver, as shown by Luck (412), although the experimental evidence is rather meagre. It should not be concluded that proteins are either stable or labile. No doubt all gradations between these two extreme types exist—perhaps in the same tissues.

Pugliese (487) investigated the protein content of the liver and muscle of fed, fasted and refed dogs with results that showed clearly a decrease in the total protein content of the livers of fasted dogs when expressed per unit of original body weight. The results were confirmed in other species, in chickens and ducks by Seitz (542), in mice by Tichmeneff (612), and in frogs by Gautier and Thiers (266), and all showed the great lability of much of the liver

protein. In addition, the experiments with frogs showed the great regenerating power of the liver.

In more recent years Addis and coworkers (7) have examined the protein content of the tissues of groups of rats starved for either two or seven days. The results of these experiments are shown in Table XV from which it is seen that although the greatest absolute loss of protein is from the carcass (mostly muscle and skin), by far the greatest percent loss is suffered by the liver. The

TABLE XV
PROTEIN LOST ON FASTING RATS

	Two Day Fast		Seven Day Fast		% Total
	mg.	% Tissue	mg.	% Tissue	
Liver	155	20	304	40	16
Alimentary Tract			254	28	14
Kidney	4	4	22	20	1
Drawn Blood			11	20	6
Heart	2	4	11	18	0.6
Brain			4	5	
Carcass	700	4	1170	8	62

Rats originally 200 gm. Protein lost expressed as mg. per 100 gm.
Addis, T., Poo, L. J., and Lew, W., (7).

alimentary tract is also seen to lose a large fraction of its protein and of this, probably the greatest loss is suffered by the mucosa—assuming that the involuntary muscle behaves like the carcass in respect to loss of protein. In general, the internal organs show a great loss in protein, and a comparison of the percent lost by the liver in seven days (40%) with that lost by the carcass in the same period (8%) is very noteworthy.

Addis and coworkers (8) also showed that the lost protein could be rapidly regenerated by feeding a diet of high protein content. The protein of the liver showed an extremely rapid rate of regeneration so that this organ began to approach its maximum size long before the other tissues regained their normal quotas of protein. The authors also noted in these experiments that the total phosphorus declined less rapidly than the N content of the liver as a result of fasting, so that the P to N ratio increased. Similar observations had previously been made by Kossel (353) and others (542, 612). These observations suggest a relative increase in nucleoprotein in the tissue as a result of fasting—perhaps a relative increase in nucleus to cytoplasm ratio (see below). Additional work (6) showed that the protein content of tissues varied with the pro-

tein content of the diet. In general, the protein assigned to the various tissues rose with increased protein (casein) in the diet.

Kosterlitz and Cramb (359) performed experiments with a view to determining the nature and locus of this labile liver protein. The data summarized in Table XVI show that the number of nuclei in the tissue are not altered by fasting although there is a great loss of weight. This loss of weight is reflected in a loss of protein and phospholipid which occur at about the same rate. Simultaneously there is also a loss of protein phosphorus which repre-

TABLE XVI

THE EFFECT OF FASTING ON THE PROTEIN CONTENT AND
OTHER LIVER CONSTITUENTS OF RATS*

	Weight gm.	Protein N mg.	Protein P mg.	Phospho- lipid P mg.	Protein N Phospho- lipid P	Rel No.† Nuclei
Fed	3.46	94.9	3.79	4.79	19.8	588
Fasted 24 hrs.	2.43	77.3	3.31	3.81	20.3	602
% loss	29.8	18.6	12.7	20.5	—	—
Fasted 48 hrs.	2.32	72.2	3.11	3.55	20.3	600
% loss	33.0	23.8	17.9	25.9	—	—

* Data calculated per 100 gm. original body weight.

† Nuclei in 10^{-3} mm.³ embedded liver \times wt. of liver.
Kosterlitz, H. W., and Cramb, I. D. (359).

sents either a loss of ribose or of desoxyribose nucleic acid but this loss of phosphorus takes place at a slower rate than the loss of protein and phospholipid. The data were, therefore, interpreted as showing a loss of liver cytoplasm. Further investigation (356) with diets of different protein content showed that the protein to phospholipid ratio remained about constant whereas the nucleic acid showed a relative increase as the protein content of the diet was reduced. In fasted rats Davidson and Waymouth (183) showed that the liver contained more phosphorus with a greater percent of this P in the form of desoxyribonucleic acid. So fasting caused a fall in the ribonucleic acid such as would be expected if the animals lost protein from both the particulate and nonparticulate part of the cytoplasm.

Kosterlitz (355, 356) also showed that there were similar losses of protein from the livers of animals on a protein free diet amounting to 16.2% in one day, 23.8% in two days, until in 28 days the loss amounted to 39.6%. Phospholipid and nucleoprotein behave as already described for the fasted animals. It appears also that the male rat can form more labile liver cytoplasm than the female (145)

and that the amount of this labile protein is proportional to the log of the protein intake (358).

Most of these results have been confirmed by Harrison and Long (291), who used the ability of a fed protein to cause the regeneration of liver protein as a method of estimating the biological value of proteins. It is of interest to note that some years previously Gautier (265) had attempted to use the regeneration of frog liver in a somewhat similar fashion.

It should be noted in connection with experiments of the type that have just been cited, that fasting an animal is not equivalent to putting the same animal on a protein free diet. In fasting the percent protein in the liver rises somewhat due to the reduction in its fat and carbohydrate content, but on placing an animal on a

TABLE XVII
LOSS OF PROTEIN AND ENZYMES FROM LIVER OF
RATS FASTED FOR SEVEN DAYS

	Loss in percent*	
	Males	Females
Protein	34	30
Catalase	43	58
Alkaline Phosphatase	43	33
Xanthine Oxidase	74	67
Cathepsin (pH 3.75)	28	33†

* Results expressed as loss of enzyme per 100 gm. original body weight.

† Probable error in this figure much higher than in other determinations.

Miller, L. L. (439).

protein free diet the carbohydrate and fat show an increase so that the percent protein suffers a decrease (541a).

It is apparent that the changes in the protein content of tissues must be responsible for the lag in attaining nitrogen equilibrium which is exhibited by animals when the protein content of the diet is changed. A direct correlation between these changes has been made by Campbell and Kosterlitz (145a) in experiments on rats kept on a protein free diet for five days. Sixty percent or more of the *extra* nitrogen excreted by these animals (TN-EN) was shown to have arisen from the breakdown of labile liver protein.

In this regard it is of interest to inquire more deeply into the nature of the protein which is lost so readily. Miller (439) found that the percent loss of the various enzymes from rat liver which occurred as a result of fasting animals for seven days was not the same. Of those investigated it was shown that the loss of the enzymes catalase, alkaline phosphatase, xanthine oxidase and cathep-

sin (pH 3.75) was equal to or greater than the loss of protein as shown by the data in Table XVII. In fact in the case of xanthine oxidase the loss of enzyme was far greater than the loss of protein. On refeeding the animals a diet containing 25% casein there was, in most rats, a prompt restoration of both protein and enzyme. The result of fasting on liver and kidney cathepsin II of another species, namely the rabbit, have also been obtained by Schultz (532a). It was shown that in a six day fast the enzyme decreased more than did the nitrogen content of the liver. Evidently there is a species difference involved or else the two types of cathepsin do not behave similarly.

Similar work has also been carried out in other laboratories, particularly with respect to the enzyme arginase. Thus it was shown by Lightbody and Kleinman (403a) that the arginase content of the liver increases with an increase in the protein intake of rats, with the enzyme showing a greater increase than the total protein of the organ. Consequently, these authors interpreted their results as showing an adaptation of the animal to a dietary change; the greater the content of nitrogen in the diet, the greater the requirement for arginase which is necessary to facilitate the formation of urea from the surplus amino acid. Seifter and coworkers (541a) also examined the behavior of the liver arginase, as well as of the D-amino acid oxidase, of rats fed a protein free diet. It was shown that the arginase activity decreased about 60% in terms of units per gram of liver nitrogen following a 14 day fast whereas the oxidase showed a decrease of 54% under the same conditions. Thus both of these enzymes showed a considerably greater decrease than did the liver nitrogen (protein) and behaved in this respect like the xanthine oxidase (and to a lesser extent like the catalase) in the experiments of Miller, and like the cathepsin II of the experiments of Schultz but unlike the cathepsin (pH 3.75) of Miller.

Therefore, insofar as can be deduced from the existing data, which is much less extensive than could be desired, it appears that the so-called labile protein is comprised in part at least, of various enzymes exhibiting labilities of varying magnitudes. The amount of the various enzymes primarily concerned with the metabolism of protein and also of other enzymes not directly related to protein metabolism depends on the protein content of the diet.

We have, therefore, from the work cited in this section a picture which shows that a part of the body protein, which appears to be cytoplasmic in nature, is exceedingly labile. The liver apparently

contains more of this type of protein than does other tissue but its total mass is greater in the carcass. Lability apparently is not an attribute of some inactive storage protein but extends to all of those enzymes which have been investigated. It has been seen that loss of liver protein is considerable on a protein free diet, and it can be calculated from the data of Addis, Poo and Lew (7) that under suitable conditions the liver can gain protein at the rate of 1% per hour. It appears legitimate, therefore, to anticipate that there will be diurnal variations in the protein content of the animal, which will bear some relation to feeding habits. That is, at least some small fraction of the daily protein intake will go to form protein temporarily to break down later so that the animal may remain in nitrogen equilibrium over any given period of sufficient length. Such changes are apparent at least in the data of Higgins, Berkson and Flock (305) obtained on rats fed only during a daily two hour period.

4. The Introduction of Amino Acid into Tissue Protein

From the evidence presented in the previous sections there is good reason to believe that a part of the dietary amino acid will become incorporated into tissue protein if only to replace the endogenous losses. The extent of such incorporation was not realized until labeled amino acids were fed to experimental animals. First indications of such incorporation were obtained by enriching the body fluids of animals with deuterium oxide (617). When this was done, deuterium was later found in the proteins in stable positions. More of the hydrogen in the dicarboxylic acids was replaced than in the other amino acids investigated.

Reference has already been made to some among the experiments of Schoenheimer, Rittenberg and others using glycine (496), tyrosine (525), histidine (609), arginine (79) and aspartic acid (662) labeled with N^{15} , and with leucine (526, 497), lysine (643, 672), and proline labeled with both N^{15} and deuterium (587). All these experiments show the unexpectedly large extent to which fed amino acid and its nitrogen may be introduced into body protein. The data are summarized in Table XVIII, whence it is seen that the minimum incorporation observed following a 3 or 4 day feeding period is 29% after arginine, with the incorporation in the case of L-lysine amounting to 66%. It should be observed that in the arginine experiment the label was located in the amidine group. A label in this position would presumably be lost rapidly in

the liver, due to the action of arginase, so that the labeling of the proteins would be less than might be anticipated.

When glycine was fed, 13% of the labeled nitrogen was deposited as glycine out of a total of 44% of the nitrogen which appeared in the proteins. In other words, 31% was in amino acids other than glycine. The total glycine fed per rat over the 3 day period may be estimated at about 500 mg., and of this amount at least 65 mg. entered the proteins. Similarly, with L-leucine it was calculated, based on deuterium analyses, that 31% of the amino acid fed was

TABLE XVIII

PARTITION OF NITROGEN AFTER FEEDING LABELED AMINO ACIDS

	Days Fed	Feces	Urine	NPN	Protein	Total Balance	References
Glycine	3	2.5	40.6	11.6	44.3	99.0	496
L-leucine	3	2.1	27.6	7.8	57.5	95.0	526
D-leucine	3	1.7	58.3	11.7	34.4	106.0	497
L-lysine	4	1.3	23.9	15.2	65.8	106.2	643
D-lysine	4	1.5	69.1	5.5	21.4	97.5	672
L-histidine	3	3.0	42.0	5.0	32.0	82.0	609
L-proline	3	2.6	39.7	12.7	59.8	114.8	587
L-arginine	3	1.1	52.2	8.2	28.9	90.7	79
L-aspartic acid*	3	1.6	43.4	5.5	39.4	89.9	662

Lysine— ϵ -N labeled.

Histidine— γ -N labeled.

Arginine—Amidine N labeled.

* To the total balance should be added 0.6% as lipid nitrogen.

introduced into the protein. Following the administration of D-amino acids much less was introduced, because in this case the N^{15} must be completely removed and then be used after reformation of amino acid.

When the actual replacements of amino acids in different tissues by labeled amino acids are compared, rather striking differences are observed. Thus, the dietary leucine replaced 24% of the liver leucine in three days, whereas in the same time only 7% of the carcass leucine was replaced (Table XII). With histidine, 29% of the liver and 8% of the carcass histidine were replaced by the dietary amino acid in the same period of time. Now one of the chief problems, with regard to the observed incorporation of amino acid, revolves about the following question: Is the incorporation due to exchange of amino acid in the protein with that in the medium without complete breakdown of the protein molecule (*i.e.*, random opening and closing of peptide bonds), or is there a complete dissolution of the protein once this process is initiated so that incorporation of amino acid represents the synthesis of a

completely new molecule? The similarity between the rates of incorporation of these two amino acids suggests a process of total syntheses. However, it should be noted that proline is apparently introduced at a more rapid rate, assuming that the proteins of the total internal organs do not behave much differently from those of the liver.¹³

If the amounts of isotope taken up by tissues are compared by assigning a value of 100 to plasma protein, then the results of a variety of studies can be compared as in Table XIX. In general, it is seen from this table that there is a consistency in behavior of any one tissue to all the L-amino acids used. The uptake by liver always turns out high, somewhat less is taken up by spleen and kidney. A large uptake is exhibited by the gastrointestinal tract and this is particularly noticeable in the methionine, tyrosine and glycine experiments where the mucosa is separated from the muscle. At the other end of the scale it is noteworthy that low uptakes are observed in muscle and skin, and red blood cells. The low uptake by the last mentioned tissue is no doubt due to the time it takes for the cell to mature and appear in the circulation. It is understandable that the levels of labeling in the different tissues are similar when the labeled amino acid introduced in the system contains N^{15} and the N^{15} concentration in the total protein nitrogen is measured, because usually the amino acid fed will suffer considerable deamination and the $N^{15}H_3$ produced will be used to label other amino acids such as aspartic and glutamic, together with their amides. Thus much of the labeling in the protein will be due to a group of amino acids other than the one fed, unless of course, the acid given is one of the dicarboxylics. The specific example of glycine has already been used to illustrate this phenomenon. When the labeled amino acid given is of the D configuration, this is all the more true, because here deamination is presumably obligatory before any of the nitrogen is incorporated into the protein.

Caution is, however, necessary in drawing conclusions regarding protein synthesis from data such as that presented in Table XIX. The data are suggestive but of somewhat limited usefulness because of the multifarious nature of tissue proteins and because of many other complicating factors which cannot be dealt with in any simple manner. The validity of making comparisons of uptake by assigning the plasma a value of 100 involves some tacit assumptions

¹³ This may be due to an erroneous estimate of the dietary proline.

concerning the nature of the processes involved. The comparison is, in the first place, good only if the amino acid composition of the different tissues concerned is approximately the same, and if the rate of introduction of the amino acid into the tissue protein is proportional to the percent of the amino acid in the protein.

Also the rate of incorporation of a labeled amino acid into a tissue protein will be some function of the total concentration of

TABLE XIX
RELATIVE RATES OF AMINO ACID INCORPORATION INTO
ORGAN AND TISSUE PROTEINS

(Plasma = 100)										
Label	N ¹⁵	N ¹⁵	N ¹⁵	N ¹⁵	N ¹⁵	N ¹⁵	S ³⁵	S ³⁵	C ¹⁴	C ¹⁴
	Gly- cine	L Leu- cine	D Leu- cine	L Pro- line	L Histi- dine	L Argi- nine	DL Methionine (1)	DL (2)	DL Tyro- sine (3)	Gly- cine (4)
Liver	79	56	77	75	78	117	80	77	58	114
Kidney		82	84	54		260	95	114	132	108
G.I.T.*	55	90	66	53		480	157	154	175	206
Spleen		67	54	47			57			81
Bone Marrow				95					0	8
Erythrocytes	25	18	24	13	16					
Brain	21						21	23	13	5
Testes		46		40				33	33	28
Heart		54		24				27		
Muscle		20	22	18				19	5	8
Skin		11	11	36	15					
Carcass	16	28	21	18		70	15		18	

* G.I.T. =Gastrointestinal tract.
 (1) Uptake 24 hours after feeding methionine (595).
 (2) Uptake 6 hours after injecting methionine (247).
 (3, 4) Uptake 6 hours after injecting tyrosine (653) or glycine (273).
 The data refer to the concentrations of labeled nitrogen or carbon in the proteins of the various tissues of rats and not to the incorporation of the specific amino acids. In the experiment with S³⁵ labeled methionine the data refer to the concentration of labeled sulfur per unit of protein sulfur. In the experiments with the nitrogen label the amino acids were all fed for three days in the diet, but in those with methionine and C¹⁴ labeled tyrosine and glycine the uptake was measured 6 or 24 hours after feeding or injecting the amino acid into fasted animals.
 See Table XVIII for N¹⁵ references.

amino acid in the tissue and also a function of the specific activity of the labeled amino acid in the tissue. If the tissue is capable of forming the amino acid from unlabeled components, or if the tissue is capable of rapidly catabolizing the amino acid then the degree of labeling may not attain the magnitude which might be anticipated. If, on the other hand, the tissue is capable of concentrating the amino acid to an unusual degree then the magnitude of incorporation may be higher than that anticipated. The anomalous results with arginine are explicable as being due to the fact that in the liver this amino acid suffers loss of the labeled group to

form urea. Since this reaction does not occur to the same extent in other tissues, this leads to a spuriously lower labeling in liver and plasma, and due to this method of expressing the results, to an apparently high labeling elsewhere. The high uptake of proline by the skin proteins was attributed to the high content of this amino acid in this tissue.¹⁴

In experiments of Tarver and Reinhardt (596) on hepatectomized dogs, it was shown that plasma protein was formed almost exclusively in the liver. This being the case, it is at first sight remarkable that all these observations show liver to have a lower activity than the plasma protein. It must mean that there exist some relatively inactive proteins in the liver and that the plasma is formed and enters the circulation before these proteins acquire much label. These experiments also show that the presence of the liver is not necessary for the incorporation of amino acid by other tissues.

Taken together the data shown in Table XIX suggest that the synthesis of protein is a completely coordinated process involving the introduction of amino acids at rates proportional to their concentrations in the tissue protein. They suggest that there may be a total synthesis of the protein molecule. But obviously the available data are insufficient to allow any safe conclusions to be drawn.

Some remarks should also be made concerning the factors which determine the percent of a given dose of labeled amino acid which will be taken up by the animals' proteins. Undoubtedly there will be differences due to the differences in rates of metabolism etc., of the individual amino acids, and obviously DL or D-amino acids will not behave in this respect like L-amino acids. The uptakes of the amino acids observed by the group of investigators using N¹⁵-labeled amino acids are generally less than those which have been found in studies in which radioactive labeling has been employed. In those of Tarver and Morse (595) only a few percent of the sulfur of methionine appeared in the first days urine following the feeding of the amino acid as compared with twenty percent or

¹⁴ The high activity of the proline in the skin can hardly arise for the reason mentioned, unless the rate of proline incorporation is disproportionately high. It might be anticipated that incorporation rates would be proportional to the concentration of the amino acid in the tissue proteins being synthesized. Alternatively, the high uptake of proline may be explained as follows: Inspection of Table XVIII shows that the uptake of proline by tissues other than the skin is low compared with that of the other amino acids. If proline were formed in these tissues (see Section IX, 3), there would be a dilution of the label in the fed proline and an apparent lowering of proline uptake, relative to those tissues in which proline is not formed. The skin is probably a non-proline forming tissue.

more of N^{15} appearing in the urine following the feeding of various N^{15} labeled amino acids as shown in Table XVIII.

The chief difference between these experiments is in the dosage of amino acid employed. Those with non-radioactive isotopic amino acids involved doses of amino acid of the order of a gram; with the radioactive amino acids doses of the order of a milligram were employed. Such tracer doses make no significant change in the amino acid content of the organism. It is to be anticipated that the larger the dose employed the less will enter the protein, that is, the larger the dose the more will be catabolized immediately by the exogenous metabolism. Thus a smaller percent of the dose will enter the proteins although the absolute amount is probably greater. For this reason it has been observed that a single dose of tyrosine is not so well taken up by proteins as the same amount incorporated into the diet (527).

This phenomenon is particularly well illustrated by the data of Sprinson and Rittenberg (574, 574a). These authors fed N^{15} labeled glycine to rats and humans on diets of different nitrogen content and found that the rate of loss, and the percent loss, of N^{15} into the urine was greatest in the animals on a high protein diet, that is, the higher the glycine in the diet the more of it was rapidly catabolized by the processes which Folin referred to as exogenous metabolism; and the higher the glycine content of the diet the smaller the percent of the dose which was incorporated into the protein. This may be expressed in another way: It is easier to saturate the anabolic system than the catabolic system by feeding amino acid. The catabolic system possessed a greater degree of flexibility than the anabolic system. Thus the Folin theory may be used equally well to explain the newer data obtained by feeding labeled amino acids as the older data obtained by feeding unlabeled amino acids or protein.

It is also probable that there are other factors concerned. Thus, it is to be anticipated that the nature of the animals used will exert a profound influence on the percent uptake; so the age and endocrine balance of the animal are of importance. It is also possible that these same influences will affect the relative proportions of the administered amino acid in the proteins of the various tissues.

5. The Rate of Turnover of Tissue Protein

From the previous section it has been seen that labeled amino acids enter the proteins of tissues with surprising rapidity but it is

quite clear that no certain information concerning rates of incorporation can be gained from data obtained at single time intervals following the introduction of the label into the animal. In order to determine rates of tissue protein turnover it is necessary to obtain complete curves showing uptake and loss of the labeled amino acid. More reliance, for purposes of interpretation, may be placed on the loss curves, which are necessarily less complicated by some

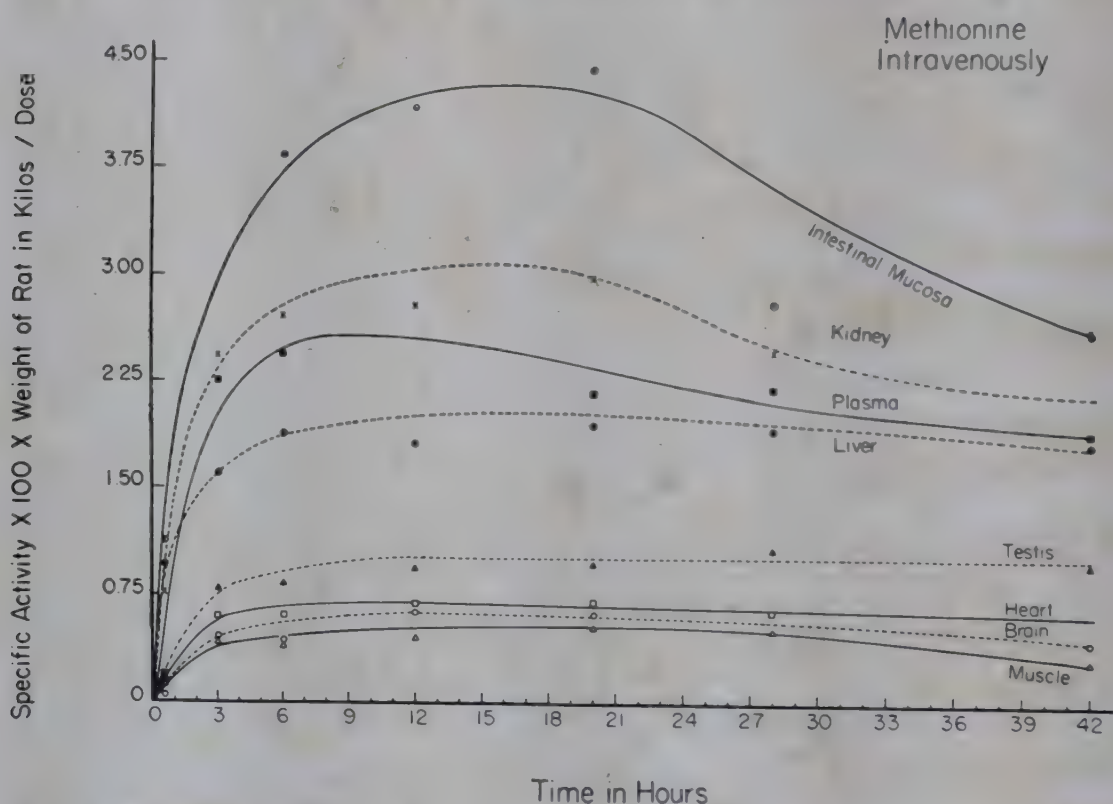


FIG. 2. Specific activity of sulfur in the protein of rat tissues immediately after intravenous injection of labeled methionine. The rising parts of the curves illustrate the incorporation of the sulfur-containing amino acids into the tissue proteins (247).

of the factors already mentioned and others which have not been considered. It should also be kept in mind that a tissue, such as liver, is losing strongly labeled protein (plasma) continuously following the introduction of labeled amino acid, whereas muscle probably does not behave in this manner. Note should be made particularly of the fact that the apparent rate of turnover will depend on the size of the pool into which the new protein is being synthesized.

More definite information concerning rates of amino acid incorporation and loss from tissue proteins has been gained by administering methionine (247, 595, 414a). In Figs. 2 and 3 are shown the concentrations of radioactive sulfur in the tissue protein

of rats at different times after the intravenous injection of a tracer dose of methionine- S^{35} (247). From these data it may be shown that the half-life of liver protein is about 4 days and of the plasma proteins about three days. In other experiments with methionine the half-life of these proteins appears to be somewhat longer (595). This is probably due to dietary differences which will be discussed

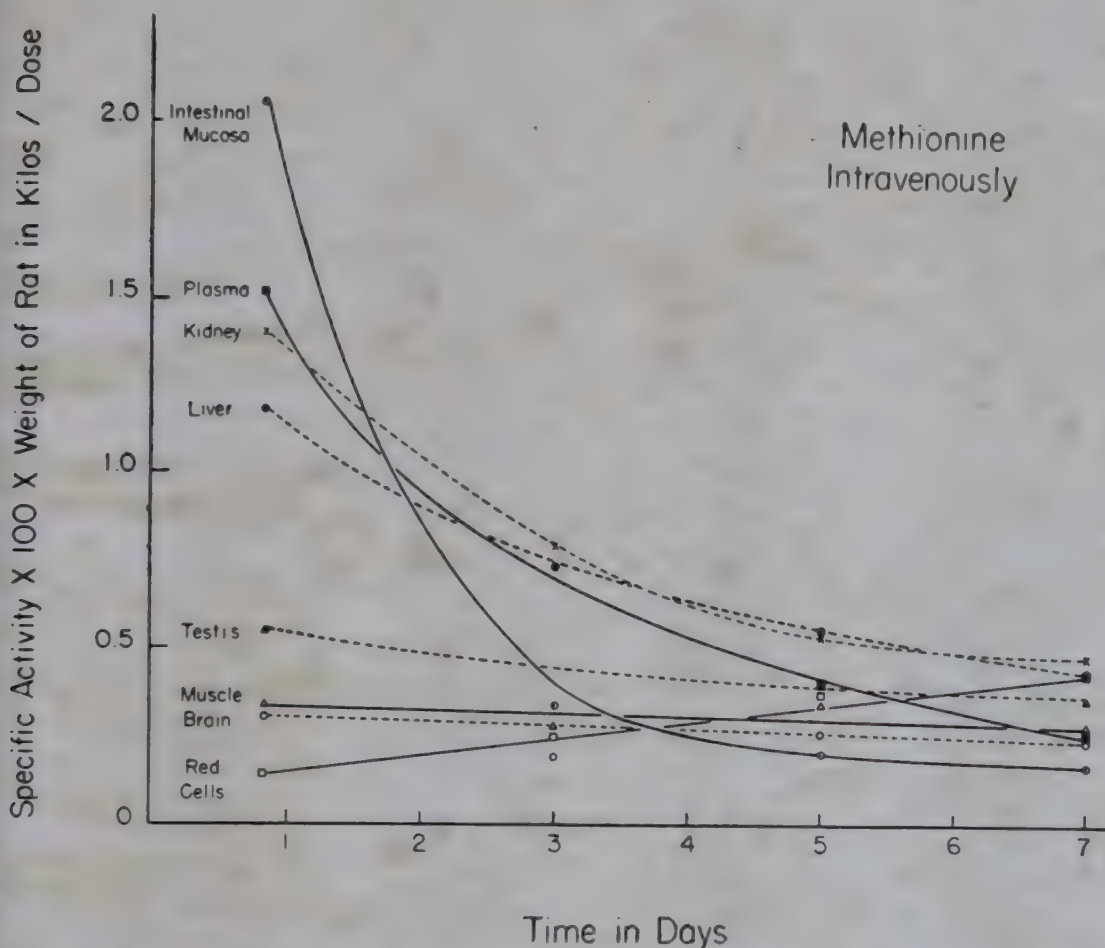


FIG. 3. Specific activity of sulfur in the proteins of rat tissues in the later stages after intravenous injection of labeled methionine. The curves in this figure illustrate the rate of dilution of radioactive methionine by inert sulfur (595).

later. Other proteins, notably those of muscle, have very long half-lives. The data obtained at an earlier date by Shemin and Rittenberg (547), who fed glycine- N^{15} to rats, were essentially similar in nature. From their data the authors found that the half-life of the liver protein nitrogen was seven days and that of the liver protein glycine was five to six days. This means that 10% or more of the liver protein is being replaced per day.

In the nitrogen experiments it was also apparent that nitrogen transfer was occurring within the organism. The carcass was gain-

ing nitrogen at the expense of the liver and other organs with high rates of turnover.

The factors which govern the rates of turnover of the tissue proteins have not been investigated. From their urinary data on the rate of excretion of N^{15} following a dose of labeled glycine given to rats, Sprinson and Rittenberg (574a) failed to demonstrate that the protein content of the diet had an influence on the rate of turnover of protein in the tissues. Their calculated value for the amount of nitrogen going into the synthesis of protein was the same regardless of whether their animals received no protein in the diet or 81% casein. This arises from their unfortunate attempt to arrive at conclusions as to what is going on inside the animal by observations made on urinary data. By making appropriate measurements directly on such tissues as liver and plasma, differences in turnover are readily discerned in changing from a diet containing no protein to one containing 25% casein (599a). As would be anticipated, on a diet of high protein content, there is a high rate of protein turnover, because under these conditions the organs contain large stores of labile protein which has a rapid turnover rate. In fact there is an approximate correlation between the rate of protein turnover in any given tissue and its labile protein content as can be seen by a comparison of the data of Table XV with Table XIX and Fig. 3. However, even when the labile protein content of the tissues is greatly reduced large uptakes of labeled amino still take place (598). Evidently, much of the protein, if not all, besides that which has been more or less arbitrarily called labile, is continually being renewed.

It is evident also that the problem of tissue protein turnover is complicated by the fact that this is not a single entity but is made up of a multitude of different proteins, all of which probably have individual rates of turnover. This is shown in a simple way by the liver studies. The protein of this organ apparently turns over more slowly than does plasma protein, yet the plasma protein is largely formed in the liver. It is, therefore, necessary that there be some precursor protein for plasma protein in the liver, which must exhibit an extremely rapid rate of turnover compared with that of the body of the organ protein. Shemin and Rittenberg have brought forward evidence to show that the situation is similar in the muscle (547).

The data concerning the apparent rate of turnover of plasma proteins will be considered in the following section.

6. The Metabolism of Blood Proteins (418, 646, 647, 451)

It is generally believed and with good reason, that fibrinogen is formed in the liver. Thus, in any condition in which there is considerable liver damage, fibrinogen shows a subnormal level. Concerning albumin and globulin, the picture obtained in liver damage is less clear, but it appears certain that a good part of both of these proteins originates in the liver. However, the increase in the globulin level of the plasma in some clinical conditions suggests involvement of extra hepatic tissues in globulin formation. The studies with hepatectomized dogs (596) also show that relatively small amounts of radioactivity can be introduced into plasma proteins in the absence of the liver and that of the small amount introduced, most is in the globulin fraction. Consequently, it must be concluded that nearly all, if not all, of the albumin is formed in the liver and that most of the globulin has a similar origin.

In a previous section it has been pointed out that there is a considerable transfer of nitrogen within the organism. The question arises, therefore, as to whether this transfer takes place via free amino acids or whether the plasma proteins function as intermediary in this process. It is known, for instance, that plasma proteins when injected serve as the sole nitrogen source. Amino acids serve similarly, so it is not shown definitely by the plasma experiments whether the plasma is broken down to amino acid in some tissue, such as the liver, prior to utilization. Experiments with phlorizinized dogs (314) suggest a utilization without complete hydrolysis. When plasma protein was fed to phlorizinized animals a large part of the protein immediately appeared as sugar in the urine, but when injected, homologous plasma was treated quite differently so that there was no detectable increase in urine sugar or nitrogen, although the injected plasma promptly disappeared from the circulation. Howland and Hawkins believed that these results showed that the protein was removed from the blood and broken down into smaller units which were reassembled to form the specific tissue proteins. However, it is probable that the rate of hydrolysis of plasma protein when injected is much less than after feeding, so that the results may be interpreted as good utilization of amino acids for purposes of maintaining nitrogen equilibrium when they appear slowly in the tissues, as compared with poor utilization when the same amino acids appear rapidly in the same tissues.

Other experiments from the same laboratory (333) show that

when the level of plasma proteins in a fasted dog is reduced by plasmapheresis—that is, by removal of whole blood and reinjecting the cells—the plasma protein is regenerated at the expense of tissue protein. Evidently, the metabolism of plasma protein is closely related to that of the proteins in the tissues. Plasma can form tissue protein or *vice versa* depending on the circumstances.

The turnover of plasma proteins has been more extensively studied than that of any other type of protein. Two chief methods have been used: Either free labeled amino acids have been injected and the incorporation and loss of the label from the proteins has been followed over a period of days or weeks; or the plasma in one animal has been labeled, as in the first type of experiment, then this labeled plasma has been removed and injected into recipient animals. It should be noted that there is one important difference between these two methods. In the first method the proteins in general are labeled, and the method is only applicable insofar as the plasma proteins are not relabeled to any considerable extent by label arising from the breakdown of previously labeled tissue protein. The second method does not suffer from this disadvantage and will, on this account, be the method of choice.

If Fig. 3 is examined it will be seen that in the rat the labeling in the plasma is down to the same level as that in muscle seven days after administering the amino acid. Consequently at this time, and thereafter, the chance of relabeling from the tissues becomes very high. From Fig. 2 it is seen that the maximum labeling is attained somewhere between the sixth and twenty-fourth hour after the dose. With these figures in mind it appears logical to use the data for the label concentrations in the proteins of plasma between the first and sixth or seventh day after feeding the amino acid in order to deduce apparent turnover rates. Assuming that the breakdown-formation of plasma protein follows the monomolecular law a linear relationship should exist when the logarithm of the label concentration in plasma protein is plotted against time, and the process may be characterized by the half-life time. When this is done for the existing data from experiments of several groups of dogs fed methionine, on two human subjects fed methionine, and on a human subject fed glycine- N^{15} , the results appear as shown in Fig. 4. From these curves it is seen that both with glycine and methionine the apparent half-life is the same, namely ten days in the human subject. In the dogs two different groups gave somewhat different figures, either five or six days, and a third group given methionine labeled plasma also yielded a value of five days.

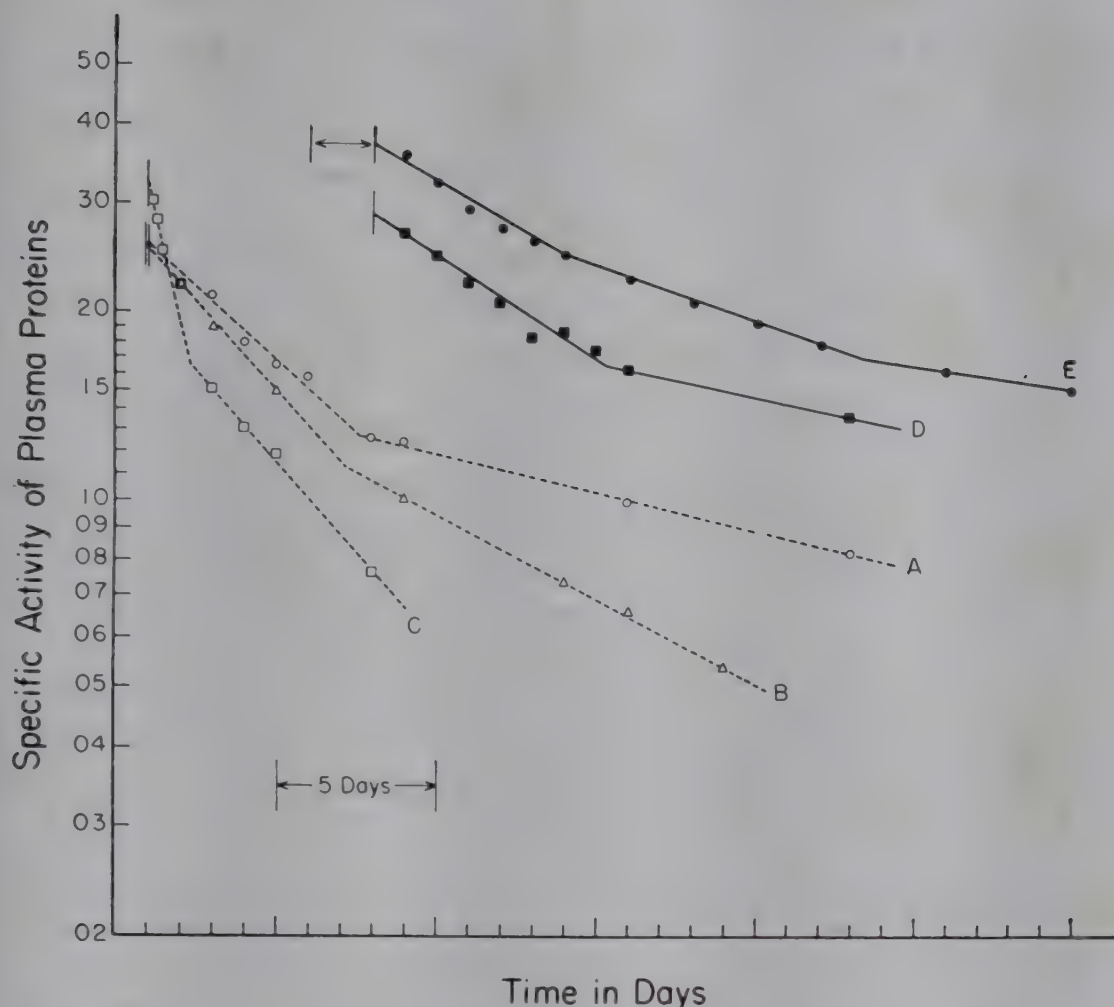


FIG. 4. Rate of loss of label from the plasma proteins of various species following the administration of labeled methionine (Curves A, B and D) or of glycine (Curve E) or of methionine-labeled plasma (Curve C). Dotted curves, experiments with dogs; unbroken lines, with human subjects. Note that the ordinate is on a logarithmic scale, and that all curves have been extrapolated back to the time the dose was administered. Glycine was fed over a two day period.*

* The activity units used for the methionine data are those of SSA, or the fraction of the dose in one milliequivalent of sulfur from the sample corrected to a standard dose of 100 counts per kilogram of animal. For the glycine data the units are in terms of N^{15} percent in the total nitrogen.

Curve A: Average results obtained in five experiments on three dogs following the administration either per os, subcutaneously, or intravenously of tracer doses (<15 mg.) of methionine. (Unpublished data from experiments on normal and cystinuric dogs [599].)

Curve B: Average results obtained in experiments on three dogs given tracer doses (<10 mg.) of methionine intravenously. (Data of Forker, Tarver and Chaikoff.)

Curve C: Average results obtained in an experiment on a 9.3 Kg. and a 6.0 Kg. dog each given 50 ml. of labeled plasma. The plasma, taken from the donor animal 10 hours after methionine, had an activity of 672 counts per minute per ml. or an SA of 6180. The SA was 313 and 523 in the larger and smaller recipients respectively four hours after the injection. The data are expressed in arbitrary units obtained from the average SSA by multiplying by a factor so that the value after one day is 2.22, i.e., the average SSA found for the plasma of dogs one day after administering methionine in experiments summarized in curves A and B. (Unpublished data of Entenman, Tarver and Chaikoff.)

Curve D: Average results obtained from two normal human subjects injected with tracer doses of methionine (Kinsell, Margen, Tarver, Frantz, Flanagan, Hutchin, Michaels, and McCallie, *J. Clin. Invest.*, 29, 238 (1950)).

Curve E: Data from a human subject fed glycine- N^{15} (Rittenberg 505a).

When plotted in the same way the rat data of Fig. 3 give a value of 2.6 days and that from the methionine data of Tarver and Morse (595) of 4.4 days. These inconsistencies within the dog data and the rat data may be assigned, tentatively, to differences in the protein contents of the diets of the animals used.

The data, shown in curve C, Fig. 4, are the average of the results obtained from two dogs which were injected with plasma proteins previously labeled in a donor animal. It is evident that the apparent rate of plasma protein turnover is five days and agrees fairly well with the data obtained by the other method (Curves A and B). However, it is seen that the turnover phase is preceded by a phase during which plasma protein is lost from the circulation at a very rapid rate. This rapid loss of plasma protein had previously been observed by other workers (235, 439a) who labeled the plasma in donor dogs by feeding lysine- ϵ -N¹⁵ or lysine- ϵ -C¹⁴. From these data, shown in Fig. 5, the apparent half-time of this process for either the plasma proteins or the separated albumin and globulin turns out to be about 11 hours.¹⁵ Similar results were obtained using plasma labeled with methionine-S³⁵ (Fig. 4).

Thus there are two processes concerned in the loss of labeled plasma from the circulation, one with a half-time of about 11 hours in the dog and the other with a half-life of 3–10 days depending on the species. Since it appears clear that the slower process is due to the catabolism of the labeled plasma, the more rapid process must be due to loss of protein from the circulation into the interstitial fluid and lymph, and perhaps into the tissue cells themselves. From the half-life data and a knowledge of the amount of protein in circulation it should be possible to calculate the amount of protein with which the plasma is in equilibrium, but since the rate of the rapid process is somewhat doubtful it does not appear profitable to proceed with such calculations until more accurate data are available. Moreover, the data so far obtained suffer from one rather serious drawback; quite considerable volumes of labeled plasma have been injected (50–200 ml). The presence of such excessive amounts of extra plasma may serve to speed up the first phase.

The rates of loss of label from antibody proteins in both active and passive immunity have also been examined (527, 296). When

¹⁵ The authors estimated the half-time of this process to be about 30 hours by drawing the curve through the data for the first 24 to 36 hours. The rate at which plasma is lost is much greater during the first 12 hours, and over this period the half-time for the process is 11 hours, or less. The slower rate in the second 12 hours is no doubt due to the change over from phase 1 to phase 2.

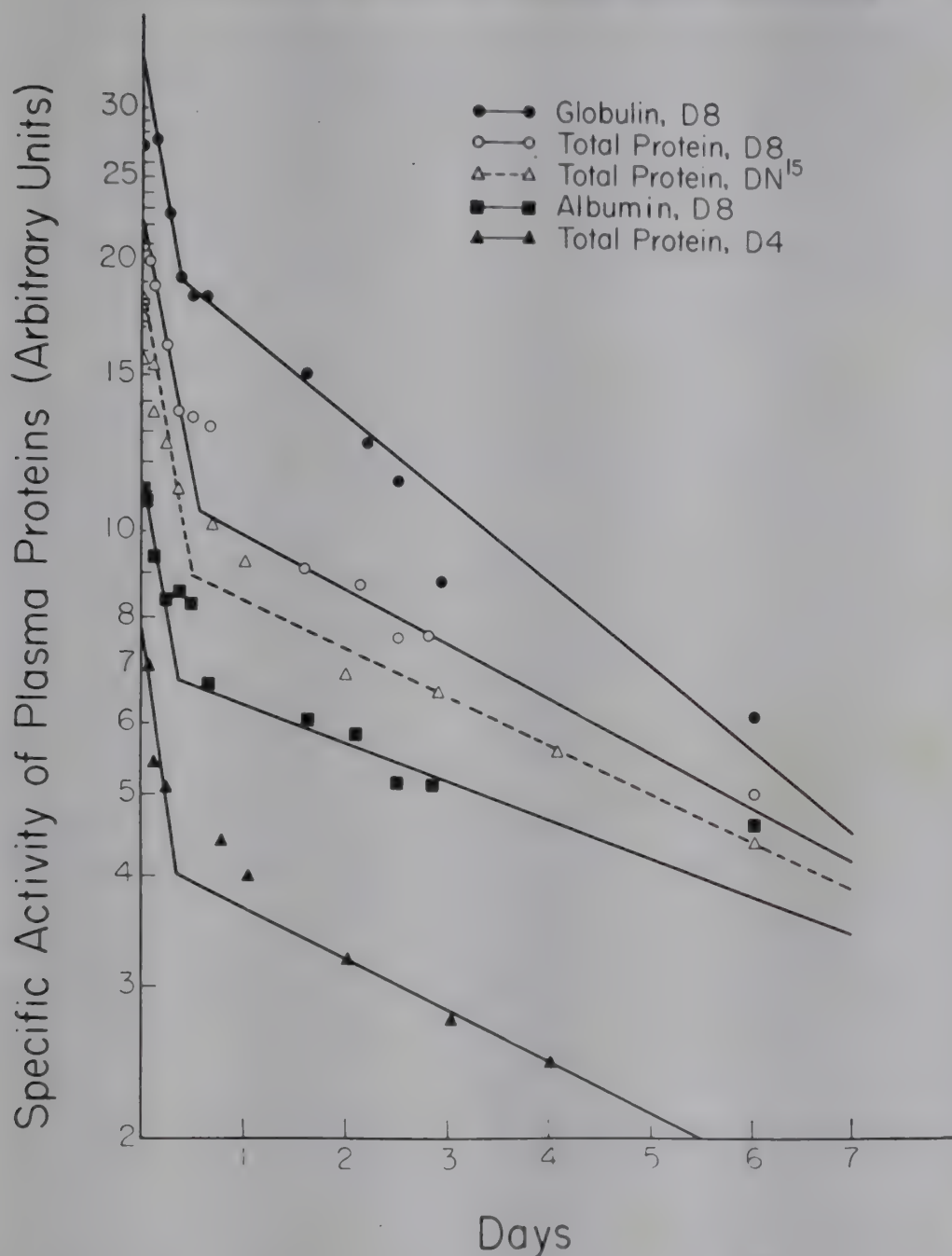


FIG. 5. Rate of loss of plasma proteins labeled with lysine from the circulation of dogs. Note that the ordinate scale is logarithmic. Data taken from Miller, Bale, Yuile, Masters, Tishkoff and Whipple (439a).

a rabbit is given a nitrogen free pneumococcus type III antigen, antibody globulins are formed. The antibody globulin is, of course, easily separated by precipitation with the specific antigen. If, during the period of antibody formation, labeled glycine or other amino acid is given the antibody globulin becomes labeled along with the rest of the plasma proteins, and at about the same rate. From these experiments the half-life of antibody nitrogen was estimated to be 12–14 days. This is longer than would have been

anticipated from the work previously considered. Incidentally, it should be noted that antibody globulin was being formed even when the amount of circulating antibody was decreasing.

In passive immunity the situation is quite different. When antipneumococcus serum was injected into a rabbit given glycine containing N^{15} no glycine nitrogen was introduced into the injected antibody. Thus, in the absence of the specific stimulant for anti-

TABLE XX
APPARENT HALF-TIMES OF PROCESSES CONCERNED IN THE
LOSS OF LABEL FROM PLASMA PROTEINS

Species	Protein	Labeling Agent	Phase		Reference
			1	2	
			Hours	Days	
Rat	Total Protein	Methionine		4.4	595
Rat	Total Protein	Methionine		2.6	247 (Fig. 3)
Rat	Total Protein	Glycine		2.3*	589
Rabbit	Active Antibody and Globulin	Glycine		12-14†	527
Rabbit	Passive Antibody			very slow	296
Dog	Total Protein	Methionine		6.4	599 (Fig. 4, A)
Dog	Total Protein	Methionine		5.2	(Fig. 4, B)
Dog	Total Protein	Methionine Plasma	(32)	5.0	(Fig. 4, C)
Dog	Total Protein	Lysine- N^{15} Plasma	11	5.4	(233) (Fig. 5)
Dog (488)	Total Protein	Lysine- C^{14} Plasma	11	4.95	(439a) (Fig. 5)
Dog (8)	Albumin	Lysine- C^{14} Plasma	11	6.9	(439a) (Fig. 5)
Dog (8)	Globulin	Lysine- C^{14} Plasma	11	3.3	(439a) (Fig. 5)
Human	Total Protein	Methionine		9.2	(Fig. 4, D)
Human	Total Protein	Glycine		10.0	505a (Fig. 4, E)

* There is some uncertainty in this value due to the paucity of the data. The authors give a half-life of 1.5 days which we fail to derive.

† The probable error in this figure is large, so that the half-life may be considerably overestimated. However, the rabbit may be on a low protein diet, and under these conditions there may be a longer half-life.

Fine, C., and Seligman, A. M., (284) have reported additional work with artificially labeled plasma.

body formation, no turnover in the antibody was found. The antibody slowly disappeared from the circulation. It may be argued, therefore, that these experiments also indicate a total synthesis of protein because there is otherwise no apparent reason why the antibody in this experiment should not have suffered rupture of peptide bonds sufficient to allow of the introduction of labeled glycine.

The data relative to the turnover of plasma proteins are summarized in Table XX. From this it becomes evident that there

may be some relationship between the body size and the apparent rate of turnover, because the rate appears to be greatest in the rat and least in man, with the values for the dog lying in an intermediate position. Thus the rate of turnover may be related to surface area rather than body weight.

Shemin and Rittenberg (550) have also used glycine labeled with N^{15} to estimate the life of the red blood cell in the circulation of a human subject. The isotope concentration in the cell protein and hemin was followed over a period of months and it was found that the hemin N^{15} concentration rose to a maximum which was attained in 25 days and maintained for 70 days. Thereafter the N^{15} concentration fell slowly. From the results the life span of the red cell was calculated to be 120-127 days in the adult male, and 109 days in the adult female (410a). A similar value (115 days) has also been found using lysine- ϵ - C^{14} to label the red blood cells of a dog (28a), and these results are in agreement with those obtained by other methods (292, 143).

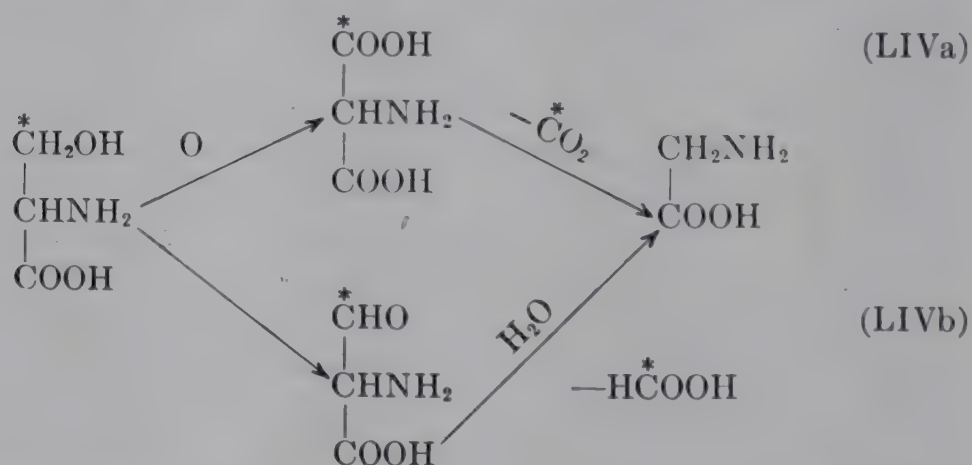
When glycine is used as a labeling agent, it is not only the protein which becomes labeled, but also the heme (porphyrin) part of the molecule (549a, 545a). In the process the carboxyl group of the glycine is lost (276a). *In vitro* there is no incorporation of the glycine into heme of non-nucleated red cells, although nucleated cells (and reticulocytes) are able to carry out the reaction. Thus duck cells incorporate N^{15} from glycine during *in vitro* incubation (545b), as do also those from patients with sickle cell anemia (545a, 410a). In this condition the label in the red cells in the circulation behaves quite differently from that in the normal individual, indicating that these cells have no finite life span. They are destroyed indiscriminately, but in polycythemia vera the cells have a normal life span. In untreated pernicious anemia the life span was shortened to 85 days in one patient. In animals whose production of red cells is stimulated by feeding phenylhydrazine, the circulating cells are likewise capable of incorporating glycine into heme (14a). Glycine appears to be the specific amino acid required in the synthesis (410a).

X. SPECIAL MECHANISMS OF AMINO ACID METABOLISM

1. Unsubstituted Aliphatic Amino Acids

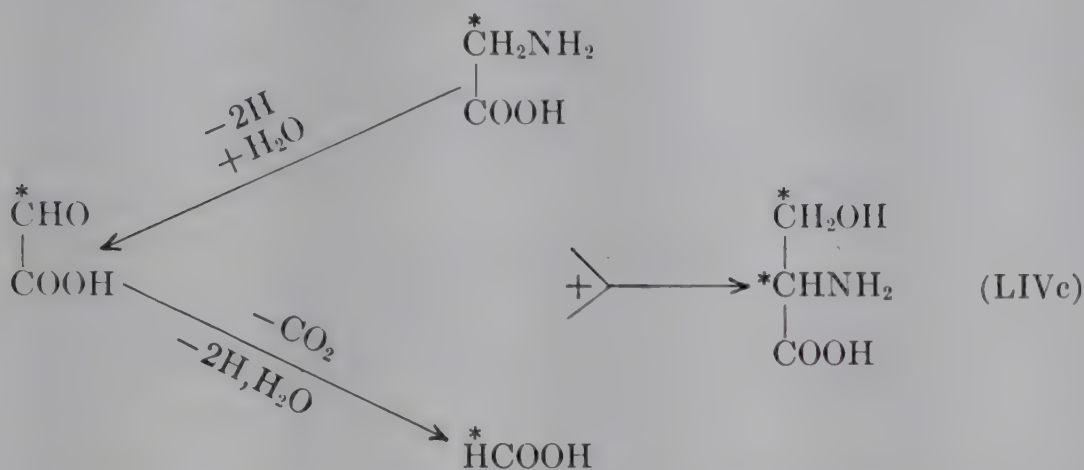
Not a great deal can be added to what has already been given in the preceding sections regarding the metabolism of the ali-

phatic amino acids, alanine, valine, leucine and isoleucine, but glycine provides an exception. Bach (22) found that the breakdown of glycine to ammonia or other specific products was difficult to demonstrate in tissue slice experiments, and concluded that it underwent condensation prior to metabolism. Additional evidence supporting this point of view has now been brought forward in that it has been shown that glycine condenses with formic acid to produce serine. The reverse reaction, namely the conversion of serine to glycine, was first observed by Shemin (545), as noted in section II, 4c, in experiments concerned with the source of glycine for hippuric acid synthesis. He concluded that the mechanism of the degradation could not proceed via the formation of aminomalonic acid—a symmetrical compound—because of the retention of the label in the carboxyl group and amino groups in the same ratio as it existed in the serine used. However, in view of the three-point attachment theory of enzyme action proposed by Ogston (470b) and supported by recent work (484a), it is not necessary to discard this theory summarily. The reaction LIVa may occur or alternatively LIVb may represent the mechanism.



The conversion of glycine to serine was shown by Winnick and coworkers (653a, 266b) who found that part of the radioactivity in the protein of liver homogenates, which had been incubated with glycine- C^{14} , appeared in serine, with little of the label in glutamic or aspartic acids or in arginine. Sakami (514) showed that glycine condensed with formate or some one of its derivatives, by isolating serine from the livers of animals fed glycine labeled with C^{13} in the carboxyl group together with formate- C^{14} . The latter label appeared in the β position and the C^{13} in the carboxyl group. The distribution of isotopes in the glycogen isolated from the liver was in agreement with this type of serine labeling. Thus, the

synthesis may proceed by the reverse of reaction LIVb, and this mechanism was supported when it was found, that, after feeding glycine- α -C¹⁴, the liver serine contained the label in both the α and β groups (558). The mechanism proposed to account for the additional finding was the following:



According to the work of Siekevitz and Greenberg (558) this reaction also proceeds rapidly in liver slices under either aerobic or anaerobic conditions.

Besides glycine there appear to be several other sources of formate. Sakami (514) found that at least one of the methyl groups of choline could be converted to formate and thus be introduced into glycine in the intact rat, and Siekevitz and Greenberg also showed that there must be formate sources besides glycine: Thus it appears that any labile methyl group is a potential source of formate and perhaps one of the ring carbons of histidine (section X, 7). Formate is not formed by the reduction of bicarbonate in the rat.

It is clear that there is no independent pathway of glycine formation from glyoxalic acid or ethanolamine (545) or by a condensation reaction involving formate (558). However, glycine may possibly be formed from glutamic acid. It has been claimed, in a preliminary communication by Arnstein and Neuberger (21a), that glycine is formed from acetate in the intact animal. However, the results *do not* show a *net* formation of glycine but only the appearance of the label in the molecule. If glycine were formed from acetate then acetate should be glycogenic. Such is not the case.

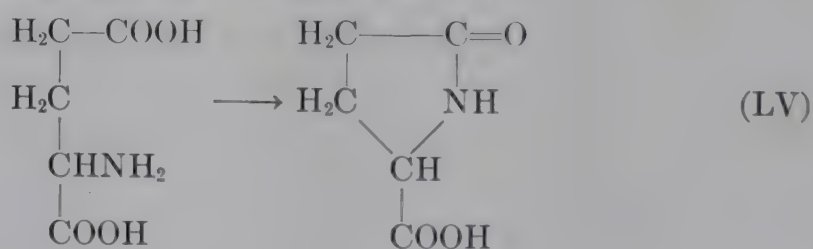
In micro-organisms glycine may be metabolized via pathways other than that through serine. Thus Barker and coworkers (32a) have shown that in *Diplococcus glycinophilus* glycine is fermented

by a mechanism which apparently involves the coupling of two molecules to give a C_4 dicarboxylic acid, which then suffers decarboxylation at both ends to give acetate as the main product.

It has also been shown that the glycine residue, or some part of it, appears in many other molecules, (545a). Mention has already been made of its conversion to part of the creatine molecule (section VI, 2), to uric acid (section VI, 3), and in connection with the latter observations it should be noted that the residue also appears in the purines of yeast (4); also glycine goes to form part of the heme of hemoglobin (section IX, 6).

2. Dicarboxylic Amino Acids

The important place of the two dicarboxylic amino acids, aspartic and glutamic acid in the transaminating system has already been detailed. When fed, D-glutamic acid behaves in quite a different manner from the L-isomer. It is largely excreted as pyrrolidone carboxylic acid (493). The catalytic formation of this compound in acid solution has been studied by Wilson and Cannan (650). The reaction may be pictured as follows:



In some bacterial species, L-aspartic acid and glutamic acid are oxidized to acetic acid, carbon dioxide, and ammonia (338). The two dicarboxylic acids are no doubt formed rapidly in the organism from pyruvate via carbon dioxide fixation and through the operation of the tricarboxylic acid cycle which provides the necessary keto acids. From α -ketoglutarate and ammonia the formation of glutamate proceeds readily as previously described (Section II, 4). This amino acid probably gives rise to aspartate by transamination with oxalacetate.

3. Arginine, Ornithine, and Proline

The conversion of ornithine to arginine in the intact animal, which must proceed rapidly if urea is to be formed from arginine

via the Krebs cycle (Section VI, 1), has been shown by Clutton and coworkers (152) who fed ornithine labeled with deuterium in stable positions and isolated deuterium labeled arginine from the tissues of the animals. In similar experiments ornithine was shown to be converted to proline and glutamic acid (511).

By several methods it has also been demonstrated that glutamic acid is formed from proline. Thus Stetten and Schoenheimer (587) showed the conversion by carrying out feeding experiments with deuterium and N¹⁵-labeled proline (Table XXI), confirming previous work with isolated liver and kidney preparations (640, 456). Also it has been observed that the so-called cyclophorase preparation is able to oxidize proline (594a), and again here the intermediate is glutamic acid. The nature of the other intermediates which must lie between proline and glutamic acid is less clear.

TABLE XXI

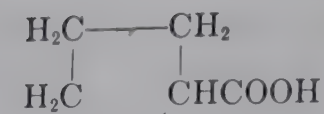
LABELING OF AMINO ACIDS IN ORGAN PROTEIN FOLLOWING THE FEEDING OF L-PROLINE CONTAINING 100 AT. % DEUTERIUM

	At. % D		At. % D
Glutamic acid	0.26	Ornithine	0.49
Aspartic acid	0.03	Proline	17.4
Arginine	0.42		

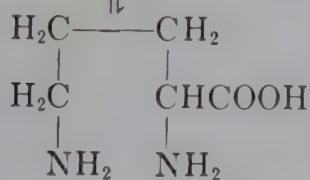
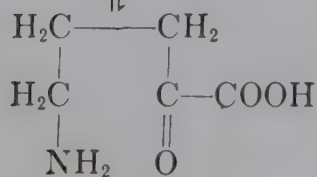
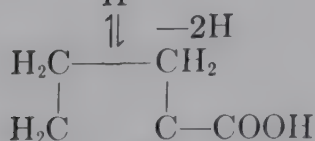
Stetten, M. R., and Schoenheimer, R. (587).

In the first place it has been shown that proline is neither metabolized through pyrrolidone carboxylic acid (see Equation LV) (640, 456) nor through α -amino- δ -hydroxyvaleric acid (456). When D-amino acid oxidase (372) or the L-amino acid oxidase of animals (70) or of bacteria (593) acts on proline the product which has been found is α -keto- δ -aminovaleric acid. The same product is formed when the D-oxidase acts on ornithine (372). However, the cyclophorase preparation apparently does not oxidise proline in this manner (594a). In this case the product appears to be the semi-aldehyde of glutamic acid, and α -keto- δ -aminovaleric acid is not attacked.

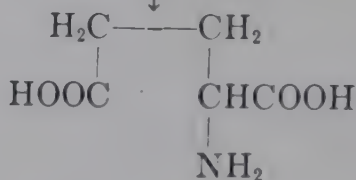
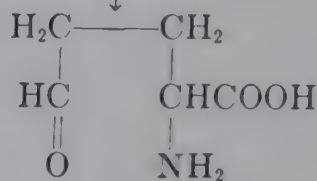
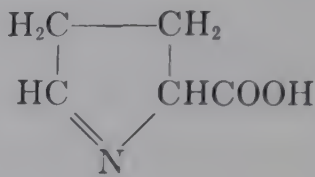
Stetten and Schoenheimer (587) also demonstrated the conversion of proline to ornithine. All the above-mentioned interconversions may be shown in the following reaction scheme (LVI) which was first put forward by these authors.



Proline



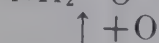
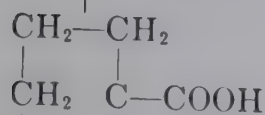
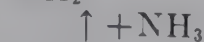
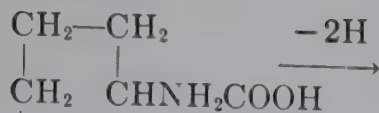
Ornithine



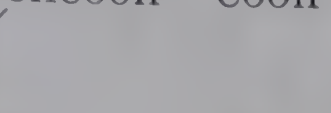
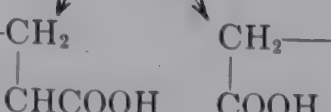
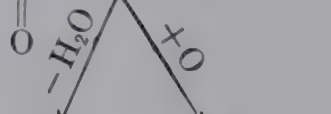
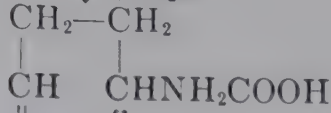
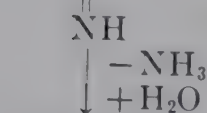
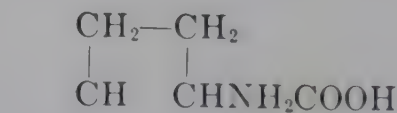
Glutamic Acid

(LVI)

Ornithine



Proline



Pyrroline-carboxylic acid

Glutamic acid

(LVia)

Another scheme (LV Ia) to explain the experimental observations so far cited and to explain the results of experiments with glycine N¹⁵ has been proposed by Shemin and Rittenberg (548). These workers found that after feeding the labeled amino acid to rats for several days, both the α - and the δ -amino groups in the ornithine of the carcass proteins of the animals attained the same concentration of N¹⁵.

According to this mechanism there is an intramolecular nitrogen shift so that the nitrogen atom in the α -position of the ornithine becomes the δ -nitrogen without being mixed with nitrogen in the medium.

However, when this scheme is examined it is seen that it really cannot explain the equality of N¹⁵ concentrations in the ornithine nitrogens, because under the conditions of the experiment of Shemin and Rittenberg unlabeled proline is continually entering the system (from the diet). This would cause a dilution of label in the δ -position relative to the α -position. Thus, although either scheme explains the observations apart from those in the glycine experiments, neither the one nor the other explains this result. It may be fortuitous and the matter needs further investigation.

In microorganisms it is apparent that similar interconversions between these three amino acids occur (599b), but the lower forms are also able to convert glutamic acid into proline and ornithine.

4. Serine and Threonine

Serine, as mentioned in Section X, 1, is readily converted to glycine and ethanolamine (584). The mechanism of ethanolamine formation appears to be one involving a decarboxylation (586, 393a). In addition, the carbon chain of serine appears as that of cysteine-cystine (586) (Section X, 5). The toxicity of the D-isomer of this amino acid has been dealt with in Chapter X (see ref. 20, 238, 239, 634).

Knoop and coworkers (345) have studied the breakdown of α -amino- β -hydroxy- ω -phenylbutyric acid and the corresponding valeric acid derivative. These amino acids were found to behave like fatty acids undergoing β -oxidation and the loss of two carbon atoms to produce phenylacetic and benzoic acids respectively. These findings are in accord with earlier ones by Knoop (343) who showed that hippuric acid is a product of the oxidation of β -phenylserine.

Serine is a component of some phospholipids, although a rather minor one (19).

5. Methionine, Cysteine, and Cystine (398, 614, 24, 250)

The metabolism of these three amino acids is quite closely related because the sulfur of methionine can be converted to that of cystine-cysteine. The latter two amino acids are presumably interconvertible in the organism.



It is generally assumed that methionine suffers demethylation to give homocysteine, and some methylated compound such as choline although this conversion lacks direct proof. However, the reverse reaction has been demonstrated by Borsook and Dubnoff (108). At any rate, the methyl group of methionine exhibits a certain lability and can be used to methylate guanidino-acetic acid (99) and ethanolamine to form creatine and choline, respectively (624). The methylation of guanidinoacetic acid differs from that of homocysteine in that the mechanism is more complex (108).

During the process of methyl group transfer none of the hydrogen atoms in the methyl group is lost. The group is transferred intact as shown by studies involving the labeling of the group with both carbon and deuterium (332). The ratio in which these labels existed in the methionine fed was maintained in the methylated products, choline and creatine, found in the body of the experimental rat used in the study. Thus, the methyl group must be transferred as such rather than in some form such as formaldehyde or formic acid.* In addition it has been shown by direct measurement with the methyl labeled compound that the methyl group of methionine is oxidized to carbon dioxide (417). This must be true, almost of necessity, since following the feeding of methionine there is no accumulation of methylated compounds in the organism although there is some excreted in this form.

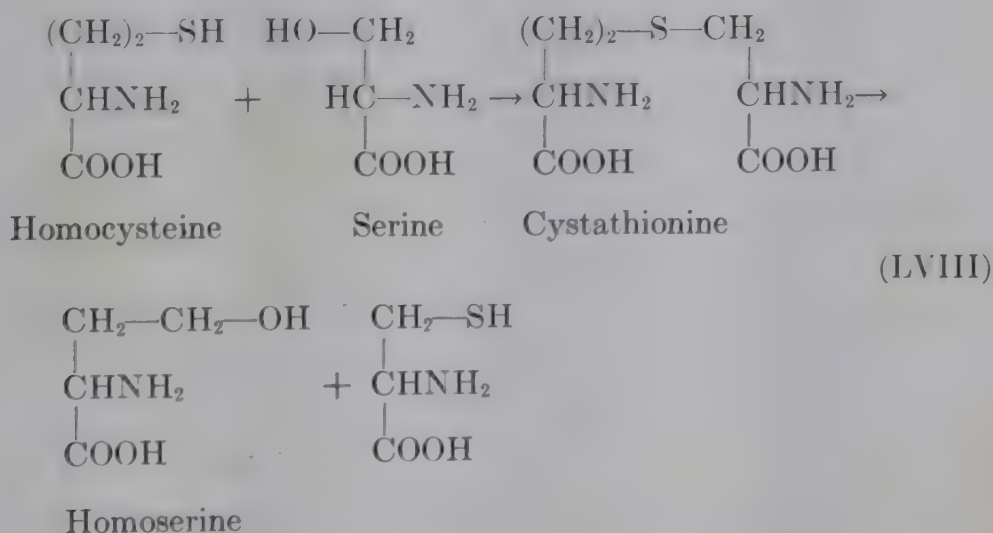
The data do not mean that the methyl group is not oxidized *in situ*, but only that the oxidation is not a prerequisite to transfer.

* It has been demonstrated recently, however, that formate is reduced to form labile methyl groups which may be introduced into methionine both *in vivo* and *in vitro* (Welch, A. D., and Sakami, W.: *Fed. Proc.*, 9: 245 (1950); Siekevitz, P., and Greenberg, D. M.: *J. Biol. Chem.* (In press).)

Formic acid may be an intermediate in the oxidation which presumably must proceed stepwise for the carbon of the labeled methyl group appears in serine (558) (Section X, 4).

As noted previously, it is possible that homocysteine suffers loss of sulfur to give hydrogen sulfide and α -ketobutyric acid or possibly α -aminobutyric acid, which has been found in the urine (185). The possible reversal of this reaction has been indicated by studies of Fromageot and Clauser (251) although the experiments of Smythe and Halliday (566) with the analogous cysteine reaction make this appear improbable. The further oxidation of homocysteine beyond the stage of homocystine is also possible (433). Neither homocysteine nor homocystine is deaminated in tissue slices (95).

In addition to the previously indicated pathways of metabolism, homocysteine also undergoes transsulfuration with the formation of cystine, *i.e.*, the conversion of methionine sulfur to cystine sulfur takes place. This was first shown by dietary methods (Chapter X), and also with radioactive sulfur by Tarver and Schmidt (597). Later du Vigneaud and coworkers (627), using C^{13} and S^{34} labeled methionine showed that there is a transfer of the sulfur from one carbon chain to another. Such a mechanism of cysteine formation had already been proposed by Brand and others (120). The following reactions apparently occur:



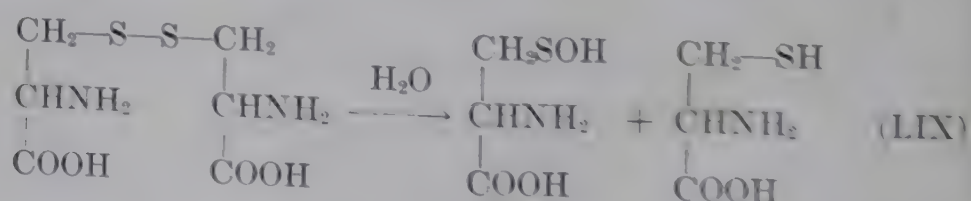
The intermediate, cystathionine, supports growth of animals on a cystine deficient diet but not those on a methionine deficient diet. *In vitro*, liver tissue converts it anaerobically into cystine (66, 18). Phosphate appears to be involved in the cleavage reaction (65).

Cystine is also formed in tissue slices from homocysteine and serine (67), although none was formed from pyruvic acid and ammonia. The participation of serine is also shown by the work of Stetten (586) who used N^{15} labeled serine. In *Neurospora* homoserine also forms methionine and threonine (605). So all the available evidence supports the position of cystathionine as the intermediate in the conversion, although its formation in animals has not been demonstrated.

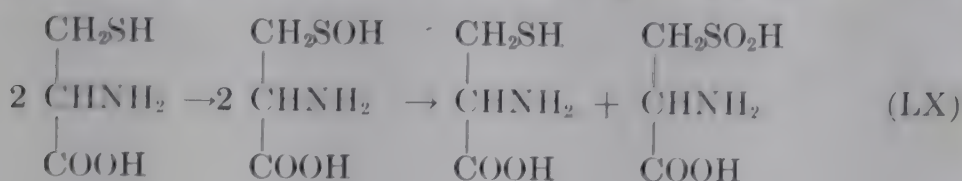
According to Carroll and coworkers (145b) L-cystathionine is cleaved to give α -ketobutyric acid as the second product of the reaction rather than homoserine (reaction LVIII). Moreover, under the conditions used DL-homoserine was converted to α -ketobutyric acid. This may explain the presence of α -aminobutyric acid in urine (185).

In *Neurospora* the reverse reaction evidently proceeds, namely the formation of cystathionine from cystine (313). Whether in animals, a cleavage of cystathionine to form homocysteine in small yield prevails cannot be deduced from the available nutritional evidence. *In vitro* the sulfide is reduced with hydriodic acid to give homocysteine thiolactone (591).

Cystine, cysteine and methionine are all readily oxidized to give extra sulfate in the urine as shown by numerous workers (663). Methionine sulfoxide is not as readily oxidized as methionine (432), so the oxidation of methionine probably does not take place directly but through homocysteine. It appears that cysteine is more readily oxidized than cystine (432), hence the oxidation of cystine may take place through cysteine formed by reduction (equation LVII) or hydrolytic cleavage:

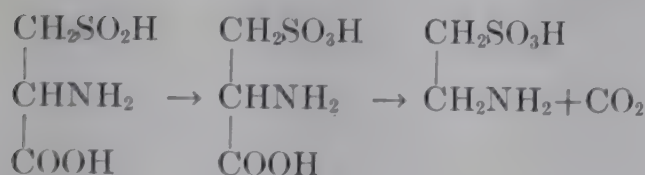


The D-isomers of both cystine (625, 12) and methionine (432) are apparently less easily oxidized than the naturally occurring isomers. These amino acids are also oxidized *in vitro* in tissue slice experiments (483, 432). Cysteine is oxidized with the consumption of three atoms of oxygen (57) and the process has been formulated as follows:



Sulfenic acid

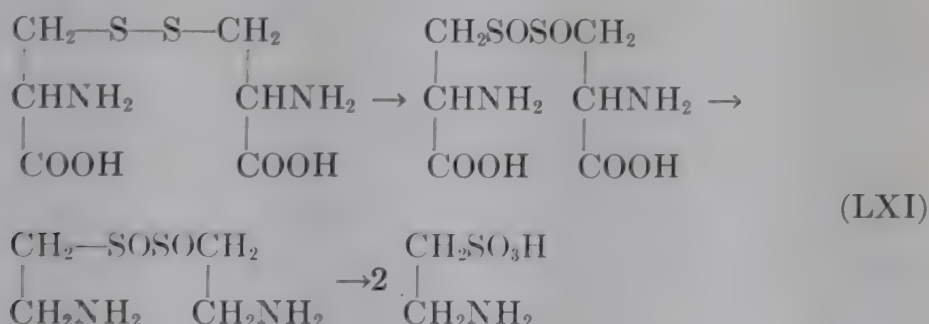
Sulfinic acid



Cysteic acid

Taurine

The decarboxylase for cysteic acid has already been mentioned in the section on decarboxylation. Cysteine sulfinic acid is also broken down to sulfite (250) and oxidized to sulfate (432). Cystine is oxidized to give the disulfoxide, which is decarboxylated and oxidized further to give taurine (433).



The distribution of cystine oxidase in tissues has been studied by Greenstein and Leuthardt (275). It is found only in liver, kidney and pancreas of mice. Cystine disulfoxide is oxidized to sulfate either directly or following reduction to cystine (432). However, these oxidation products of cystine as well as cysteic acid (648, 434) and taurine (521) are less easily oxidized than cystine and cysteine (432), consequently the major part of the cystine-cysteine metabolized may undergo oxidation following the loss of H_2S or after deamination. Hydrogen sulfide is readily oxidized to sulfate (192).

The formation of taurine is of interest in its relation to the production of taurocholic acid—a bile constituent in species such as the dog, cat and rat. Extra taurocholic acid formation from taurine may be demonstrated in depleted animals if cholic acid is given

(630). Cysteic acid forms taurocholic acid better than does cystine disulfoxide (631), and homocysteine better than homocystine (632).

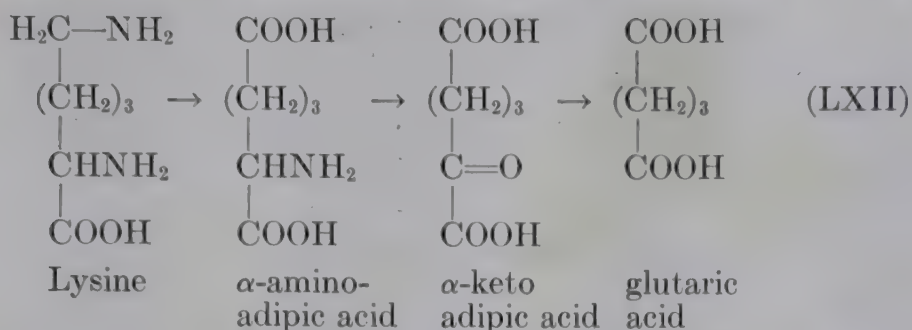
The excretion of mercapturic acids, the acetyl derivatives of cysteine, has already been described in the section on acylation. There is also, in some dogs (119) and human subjects (396), a cystinuria in which cystine itself is excreted in the urine in relatively large amounts. This may give rise to the calculi which led to the discovery of cystine by Wollaston (656). The condition is rather rare although the excretion of some cystine in the urine is a normal occurrence, the amount being of the order of 30 mg. per day (431).

Surprisingly enough, the feeding of extra cystine is without effect on the cystinuria of either the humans (15, 123, 400, 401) or the dogs (304). Methionine, on the other hand, is in part excreted as extra cystine (122, 400) as is also homocysteine but not homocystine (121, 304). Cysteine behaves like homocysteine and methionine (123, 303, 411).

When fed methionine labeled with radioactive sulfur, cystinuric dogs excrete cystine similarly labeled (599) and with a larger radioactive concentration than in the cystine excreted by the normal animal. In general, the results obtained with the cystinuric indicate either an increased formation in or loss of cystine through the kidney. A decreased rate of the oxidation of cysteine to sulfate might result in an extra formation of cystine.

6. Lysine

This amino acid is different from the others in being completely resistant to deamination as shown by the experiments of feeding D and N¹⁵ labeled lysine, not to mention all the negative results obtained using amino acid oxidase and transaminase. In the isotope experiments the lysine appears in the tissue proteins with the same D/N ratio as in the material fed (Table XII). Also, when labeled ammonia is fed, lysine contains the only α -amino nitrogen which is not replaced to any extent with that of the ammonia fed (244) except that of threonine. Concerning the mechanism whereby this amino acid is broken down, it is known now, from studies of Borsook and coworkers (97) with L-lysine- ϵ -C¹⁴, that in guinea pig liver homogenates the amino acid is broken down through the stages of α -amino adipic acid to glutaric acid. In *Neurospora* but not in rats α -amino adipic acid appears to be converted to lysine (444a, 266a).



The dicarboxylic amino acid was observed to be deaminated only slowly. D-lysine was not broken down in this fashion.

7. Histidine

Histidine is only slowly deaminated by the enzyme systems previously described, yet its α -amino N is replaced when isotopic ammonia is fed (528). The same experiments show that the N of ammonia is not introduced into the ring of histidine, *i.e.*, the ring is not synthesized by rats. Edlbacher and Grauer (197) claim that there is a specific amino acid oxidase in liver but not in kidney which acts only on L-histidine. However, the rate of deamination is slower than the breakdown of the imidazole ring which is effected by histidase; so that the enzyme is probably of little significance. Moreover, the existence of this enzyme may be questioned since the results might also be explained on the basis of the presence of histidase and glutamic acid dehydrogenase.

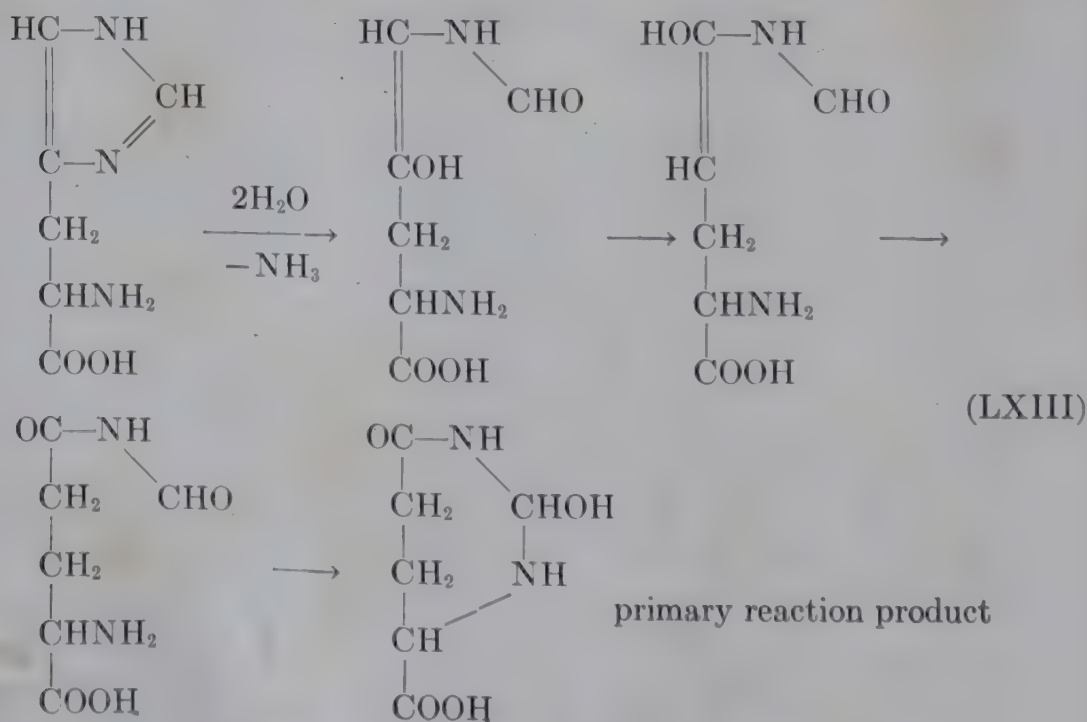
The imidazole nitrogen of histidine does not appear to be directly utilized for any special purposes such as creatine or arginine formation, although it may specifically give rise to urea (609).

The L-isomer is apparently converted into carbohydrate in the animal. This is understandable because Edlbacher and Kraus (200) have shown that the amino acid is converted into glutamic acid or rather a substance which will form glutamic acid on hydrolysis with acid. Under similar conditions L-histidine and L-glutamic acid give rise to similar increases in glycogen in the liver (227). When fed in large doses to dogs histidine leads to the excretion of non-imidazole nitrogen of unknown nature (193).

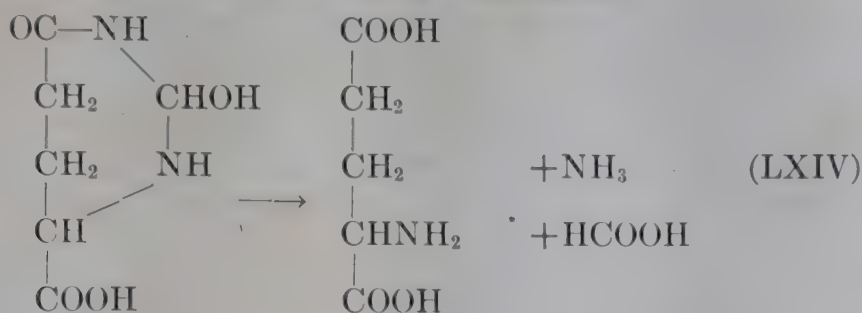
Histidase, the enzyme probably responsible for the major part of the breakdown of histidine, was discovered simultaneously by Edlbacher (194) and by György and Röthler (282). It has no action on urocanic acid (see below) and methyl histidine (200). The best source of the enzyme appears to be cat liver (200) although it is present in the livers of all vertebrates examined. The

concentration of enzyme in other tissues is quite low, so that L-histidine is broken down in liver tissue slices from the rat but not in kidney slices (228). Its preparative purification has been described by Walker and Schmidt (636). The enzyme may contain a heavy metal since its iron content is greater than can be accounted for on the basis of contamination with proteins known to contain this metal; moreover, its action is inhibited by cyanide. It is also inhibited by D-histidine and by many histidine derivatives as well as guanidine bases (195).

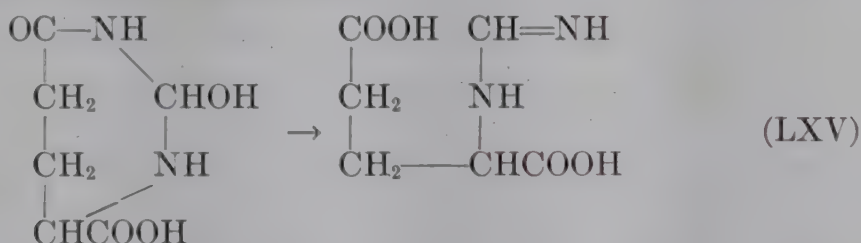
The histidase reaction proceeds anaerobically without production of CO_2 and with the formation of ammonia. The nature of the primary reaction product is unknown, but on treatment of this substance with hot concentrated hydrochloric acid, L-glutamic acid and formic acid are produced (200). During the enzymic reaction amino nitrogen disappears (200, 442) and this only reappears after the primary product is hydrolyzed. Hydrolysis with acid or alkali also leads to the production of two moles of ammonia (200), although the primary reaction leads to the formation of only one (442, 201). The appearance of the ammonia coincides with the disappearance of reactions for the imidazole ring (200). The evidence just cited suggested to Edlbacher and Neber (203) that the primary reaction took place in the ring and the α -amino N was not split off by enzyme reaction but was simply blocked. They therefore, proposed the following series of reactions to account for the results:



During the subsequent acid or alkali hydrolysis:

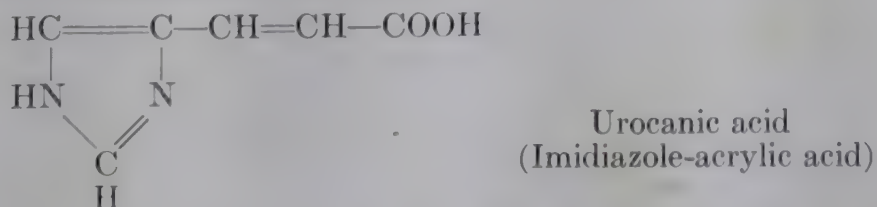


There are certain objections to this scheme. In the first place, there is no described rearrangement similar to the one postulated. Moreover, Walker and Schmidt (636) found no change in pH during the reaction but noted the formation of a group with $\text{pK} = 4.2$. The reaction product does not account for the last finding. It might change to formamidino-glutamic acid, which presumably would have the requisite properties:



Alternative mechanisms to account for the formation of formamidino-glutamic acid were proposed by Walker and Schmidt (636) but so far it has neither been shown that the primary product of the histidase reaction is converted to glutamic acid nor that it is metabolized at all by the animal organism.

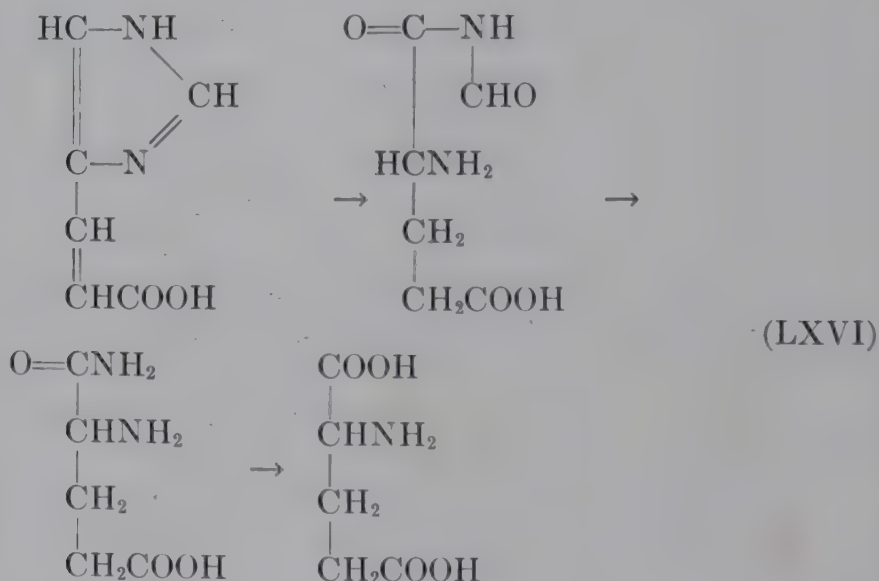
Histidine in massive doses is converted by some dogs and rabbits into urocanic acid, a result first shown by Jaffé (317) and confirmed by Kotake and others (366). The substance was found by Hunter (315) to be imidazole acrylic acid.



Evidently this substance is not on the main path of histidine metabolism in animals because it is not easily broken down in the guinea pig, rat and rabbit (199, 182). However, it may be metabolized by the dog (351). It is not formed from D-histidine (352) nor from imidazolepyruvic acid (366).

Raistrick (490) showed that urocanic acid was formed from histidine by the action of some types of bacteria and this was confirmed by Darby and Lewis (182), but bacterial action in the gut is apparently not altogether responsible for its formation in higher animals. Both Kotake and Konishi (366) in dogs and Kiyokawa (337) in rabbits obtained the substance when histidine was injected.

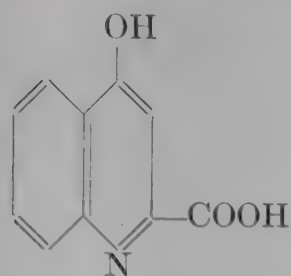
Urocanic acid is broken down by an enzyme, urocanase, which occurs in the livers (362, 204, 196) of all species examined. The enzyme is different from arginase and histidase, differing from the latter in not being inhibited by D-histidine and in not acting on histidine (204). The product of the reaction after hydrolysis with alkali is L-glutamic acid, although the primary product contains two atoms of nitrogen (196).



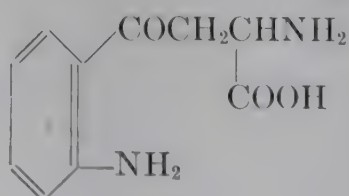
Histidine is a component of carnosine, β -alanyl histidine, which is an important constituent of the muscle in some species (621). Anserine, a similar peptide of methyl histidine, can evidently be formed by transmethylation, although slowly (517). The function of neither of these peptides is known.

8. Tryptophan

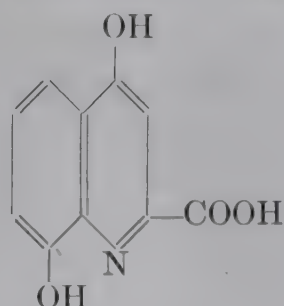
As a result of feeding tryptophan, there have been found three metabolic products in the urine:



Kynurenic acid
4-hydroxyquinoline-
2-carboxylic acid



Kynurenine



Xanthurenic acid
4,8-dihydroxyquinoline-
2-carboxylic acid

Kynurenic acid was the first of these compounds discovered (403). It is a product of the incomplete metabolism of tryptophan in the dog, coyote, guinea pig, and rabbit, but not in the cat, fox or wolf (267). It also appears in human urine (365) and has been found in the bile of some species (363, 170). The correct structure was first given by Homer (310), and a mechanism for its formation was proposed by Ellinger and Matsuoka (211).

Kynurenic acid is formed when the liver is perfused with blood containing tryptophan or indole pyruvic acid (426), but D-tryptophan does not lead to its formation (427, 48). Indole pyruvic acid and indole lactic acid also give kynurenic acid in feeding experiments, although the yield is less than when tryptophan itself is fed (426, 37). The acid, is not easily metabolized by the dog (363, 170), cat (267), or rabbit (363, 47). Consequently, it may not be a normal intermediate in tryptophan metabolism.

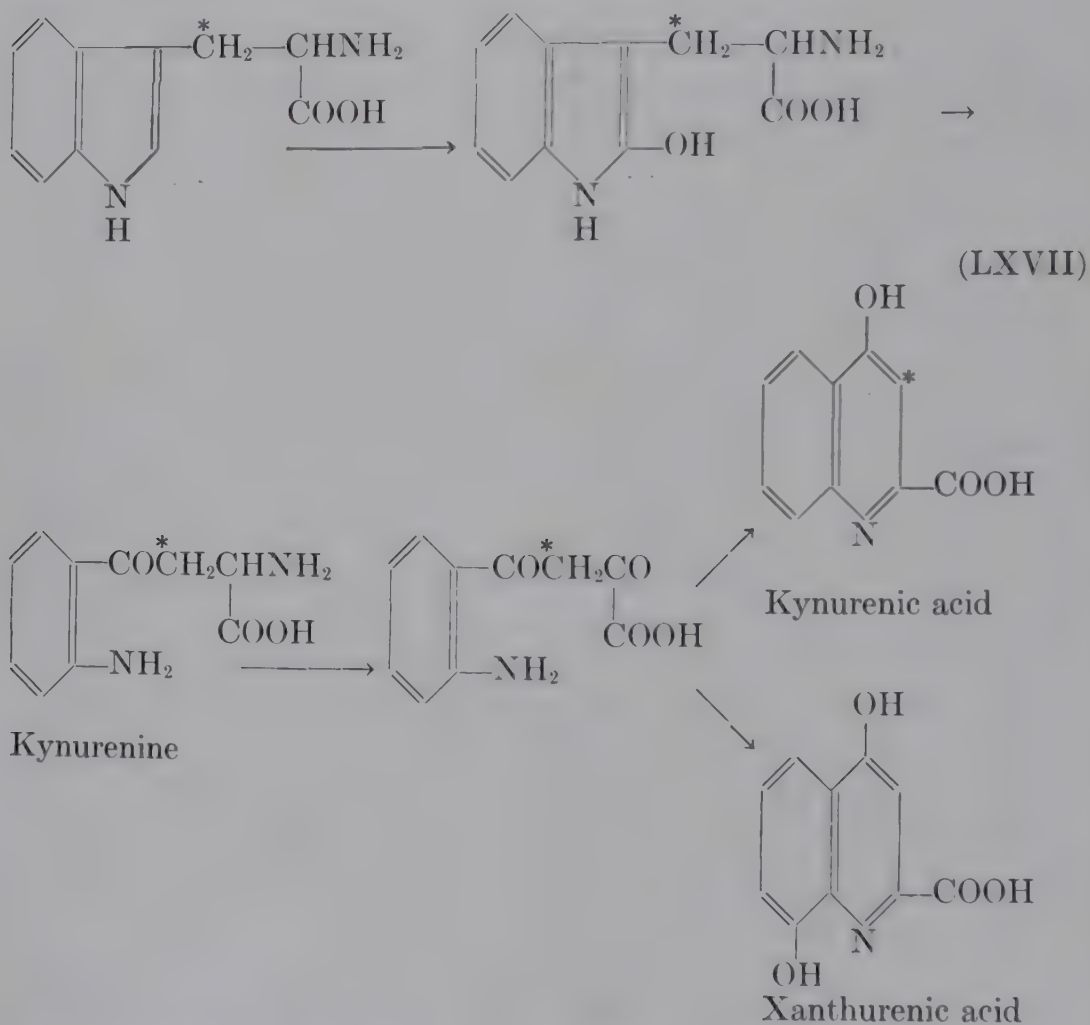
When very large doses of tryptophan are given to rabbits, kynurenine also appears in the urine (428). It is thought that this substance is an intermediate in the formation of kynurenic acid. It increases in amount relative to kynurenic acid when animals are fed a diet of polished rice, that is, when the animals are deficient in B vitamins (364). When D-tryptophan is fed it appears probable that the D-form of kynurenine appears in the urine (369, 94).

The third tryptophan metabolite was discovered by Masajo (424) in the urine of animals fed high protein diets. Later the same pigment, xanthurenic acid, was found in the urine from pyridoxine deficient rats (390, 391) and to a lesser extent in deficient dogs' urine (245). In such dogs, and even normal animals, xanthurenic acid is toxic (21) and is excreted unchanged, whereas, in normal rats it is metabolized. It is evident that there is some difference in the metabolism of tryptophan in the two species. The substance is also found in pigs' urine (146) and in that from mice (440). In

the latter species, high protein diets are toxic when there is a pyridoxine deficiency.

Kynurenine, but not kynurenic acid, gives rise to extra xanthurenic acid in the rat (499), so xanthurenic acid is not formed from kynurenic acid. Neither indole pyruvic acid nor indole lactic acid give xanthurenic acid and D-tryptophan likewise gives negative results.

In view of these facts, the metabolism of tryptophan may be represented as follows (133):



It is improbable that the major pathway of tryptophan metabolism passes through the stages of kynurenic acid or xanthurenic acid although the normal metabolism probably proceeds through kynurenine or some similar derivative. Kynurenic and xanthurenic acids may represent more or less blind alleys in catabolism.

In addition to the products already noted nicotinic acid may be formed from tryptophan. The demonstration of this conversion and the investigation of its mechanism has been made by a wide variety of methods, namely nutritional, with mutants of *Neuro-*

The conversion of the anthranilic acid derivative to nicotinic acid in liver slices has also been demonstrated (533a). In accord with these reactions and reactions LXVII are the results of Heidelberger and coworkers (296a, 296b) who employed tryptophan- β -C¹⁴ and tryptophan-3-C¹⁴. With the ring labeled tryptophan-3-C¹⁴ they found that the label appeared in the carbamido group of the N¹-methyl nicotinamide excreted by the experimental rats, but with β -labeled tryptophan no label appeared in the nicotinic acid derivative. However, in this case when the compound was administered to rabbits and dogs the label appeared in the place indicated in the kynurenic acid excreted by the animals (reactions LXVII).

It has also been shown by Sanadi and Greenberg (514a) that when the β -labeled tryptophan is fed to phlorizinized rats the urinary glucose becomes labeled in such a way as to suggest the intermediary formation of a C₂ compound. The formation of a compound giving rise to an acetylating agent was also shown by these workers by feeding *p*-aminobenzoic acid along with the labeled amino acid. The acetyl group in the excreted conjugate was found to be labeled. From this data it appears that the 3-hydroxykynurenine is deaminated and decarboxylated to produce 2-amino-3-hydroxybenzoylacetic acid, which then splits to give the acetylating agent and 3-hydroxyanthranilic acid. The data obtained by the degradation of the glucose do not suggest that tryptophan is catabolized in the rat by being broken down to serine, alanine or pyruvic acid by the operation of the tryptophanase reaction (see later).

Ellinger and coworkers (211a) reported that quinolinic acid relieved nicotinic acid deficiency in the rat and that this substance also increased the N¹-methyl nicotinamide excretion. More recently Henderson and other workers have brought forward evidence from studies with *Neurospora* (296e, 601) and rats (296c, e) that this acid, 2,3-pyridine dicarboxylic acid, is an intermediate in the conversion of 3-hydroxyanthranilic acid to nicotinic acid. The dicarboxylic acid appears in rat urine following the administration of either tryptophan or the anthranilic acid derivative, and it is evidently the labile precursor of nicotinic acid which had previously been found in urine (560a). Moreover, the hydroxyanthranilic acid is converted to nicotinic acid in liver slices and homogenates (533a, 296d). 3-Hydroxyanthranilic acid also produces quinolinic acid in liver slices (533b).

An increase in quinolinic acid excretion occurs in all animals tested when tryptophan is fed; this includes rat, man, guinea

pig, calf, lamb and pig (296f). The largest percent excretion of tryptophan in this form occurs in the rat; much lower conversions are shown by other species.

In *Neurospora* (602) and in some bacteria (272, 567) kynurenine is also converted to anthranilic acid and hence to indole and tryptophan. When the conversion to tryptophan occurs, it has been shown that the carboxyl group of the anthranilic acid is lost (470a). Beyond this there is no further information concerning the mechanism of indole formation, but with regard to the conversion of indole to tryptophan there is experimental evidence from several sources. Tatum and Bonner (600, 601) showed that the synthesis proceeds in *Neurospora* by a reaction involving serine as well as indole. They found that DL-serine is only one-half as effective as the L-isomer. Evidently the same type of reaction occurs in bacteria such as *E. coli*. A simple dehydration between the hydroxyl group of the serine and indole may be involved.

It has also been found that the enzyme system responsible for the synthesis in *Neurospora* has pyridoxal phosphate or a related compound as one of its components (616b). The requirement for synthesis of tryptophan in *Lactobacillus arabinosus* is similar (533).

However, long before the synthesis of tryptophan from indole was known, the work of Hopkins and Cole (311) had shown that tryptophan breaks down anaerobically in bacteria (*e.g.*, *E. coli*) to give indole. Aerobically indole propionic acid may be produced (657). Some bacteria act only on the L-form of the amino acid, and this knowledge has been used by Majima (419) to prepare D-tryptophan. Happold and Hoyle (288) and others (28, 658), showed that tryptophan is the specific substrate for the enzyme, tryptophanase. No indole is produced from indole derivatives with the following substituents in the β position: aldehyde, carboxylic acid, acetic acid, propionic acid or acrylic acid. In *E. coli* indole production is inhibited by the addition of serine but alanine has no effect on the reaction (601).

This breakdown of tryptophan has been investigated by several groups of coworkers, thus Wood and coworkers (660) have shown that the enzyme responsible for the breakdown in *E. coli* has pyridoxal phosphate as one of its components. These workers brought forward evidence to show that neither serine nor alanine is produced as the primary product in the degradation but that the products are pyruvic acid and ammonia. Also no oxidative step is involved

in the breakdown; so it appears that the breakdown reaction does not proceed by the same pathway as the synthetic reaction unless the actual intermediate concerned is aminoacrylic acid. Dawes, Dawson and Happold (183a), on the other hand, showed that alanine may be involved as a result of experiments in which further oxidation of the primary product was inhibited by mepacrine.

Thus it is clear that there are many possible pathways by which the breakdown of tryptophan may be effected; and it is impossible to say at the present time which one is the most important in the animal organism. It is also clear that there may be species differences involved.

It should be mentioned that the eye coloring in some insects is concerned with tryptophan metabolism in particular with the metabolism of kynurenine and 3-hydroxykynurenine (146a).

9. Phenylalanine and Tyrosine

The breakdown of these two amino acids has been investigated by numerous workers because there exist various metabolic anomalies connected with the process. The best known of these is alcaptonuria, in which homogentisic acid (2,5-dihydroxyphenylacetic acid) is excreted. The other three conditions are: (1) an extremely rare condition known as tyrosinosis, (2) phenylketonuria which is found in some mentally deficient individuals, and (3) a modification in the metabolism in ascorbic acid deficiency.

Oxidation of phenylalanine to form tyrosine has been shown in a large number of ways:

1. Livers perfused with phenylalanine form tyrosine (214).
2. Phenylalanine replaces tyrosine in the diet (see Chapter X).
3. Phenylalanine labeled with deuterium in the ring formed tyrosine which retained the label (447).
4. In tyrosinosis, fed phenylalanine gave rise^a to extra tyrosine in the urine (430).
5. Ascorbic acid deficient infants excrete tyrosine after feeding phenylalanine (394).

It is therefore quite clear that phenylalanine is oxidized to tyrosine. The oxidation occurs in the liver (58), and in spite of ample tyrosine in the diet (447). In bacteria the oxidation is prevented by giving β -2-thienylalanine (42). Certain mutants of *Neurospora* are not capable of synthesizing tyrosine from phenylalanine (559). A different type of oxidation has also been found with phenyl-

alanine, which leads to the excretion of the corresponding keto acid in the urine (367, 543, 148) together with smaller amounts of *p*-hydroxyphenyllactic acid. There is apparently a greater production of the keto acid following D-phenylalanine than when the natural isomer is given. A certain amount of decarboxylation of the keto acid also takes place so that some phenylacetic acid (as phenaceturic acid) also is found in the urine. It is possible that the yield of phenylpyruvic acid following L-phenylalanine may depend on whether the amino acid is given in basic solution or not (241).

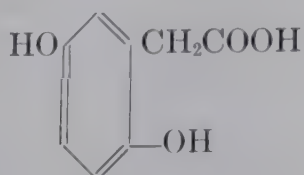
The oxidation of both phenylalanine and tyrosine has been extensively investigated in tissue slices by various workers (53, 670, 230, 381). It has quite generally been observed that the D- and L-isomers of these two amino acids behave differently. With the D-isomer the primary reaction is one of deamination which takes place most rapidly in the kidney and is unaffected by cyanide. That of L-tyrosine proceeds best in liver with the ultimate uptake of four atoms of oxygen per mole and this oxidation is sensitive to cyanide. The products are apparently acetoacetic acid and carbon dioxide. *p*-hydroxyphenylpyruvic acid is also oxidized by liver with the production of acetoacetic acid (523). Acetoacetic acid formation has also been observed to take place from the aromatic amino acids in liver perfusion experiments (216, 214) and in tissue slices (208). When fed, L-tyrosine causes an increase in keto acid in the urine (243, 635).

It has also been claimed that tyrosine breakdown proceeds with the formation of alanine (232). However, in the phlorizinized dog neither tyrosine nor phenylalanine are converted to extra glucose, although tyrosine appears to result in the formation of extra liver glycogen. If cleavage to give alanine were a prominent metabolic pathway then the demonstration of the conversion of the aromatic amino acids to extra glucose should be possible.

a. The 2,5-oxidation system

In 1859 Boedecker (91) discovered the metabolic anomaly known as alcaptonuria, in which the urine darkens on standing in contact with air. The darkening proceeds more rapidly in alkaline urine. Individuals with this condition are otherwise normal although when the condition is of long standing there may be pigmentation of cartilage (ochronosis). It is evidently transmitted by a single recessive gene (264). The structure of the substance re-

sponsible for the darkening was first shown by Wolkow and Baumann (655) to be:



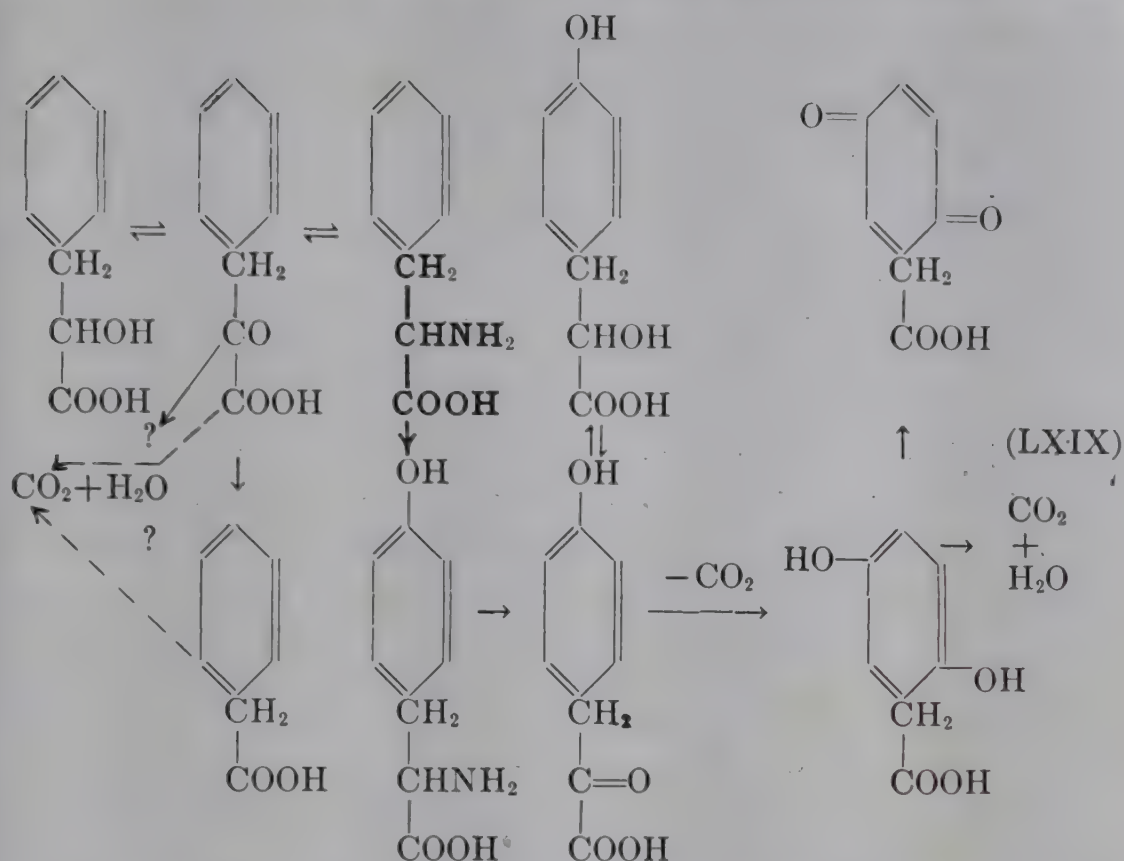
Homogentisic acid
2,5-dihydroxy phenylacetic acid.

It is readily oxidized by Fehling's solution and by ammoniacal silver. It may appear in the urine in amounts up to several grams a day. It has been shown that tyrosine (655) or phenylalanine (226, 477, 478) increase the output although tryptophan proved ineffective in this respect. In fasting the excretion is reduced, but never to zero.

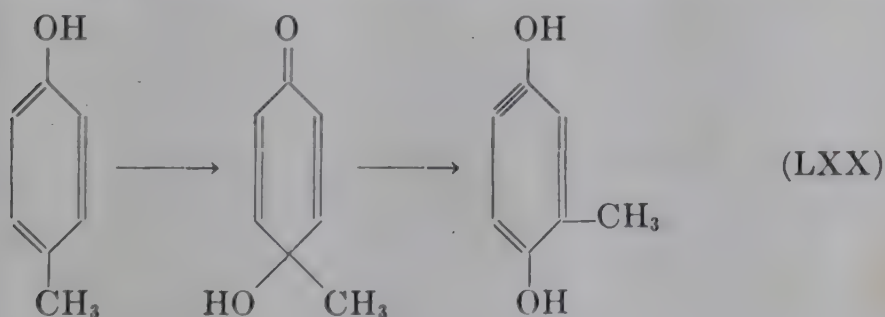
Homogentisic acid has also been found in the urine following the persistent feeding of large doses of phenylalanine or tyrosine in the rat (226, 477, 138, 383, 1, 534) and in the mouse (241). Also, in guinea pigs deficient in ascorbic acid the acid appears in the urine (540, 539). In these animals the phenol disappears from the urine as soon as sufficient ascorbic acid is fed, but in the human with alcaptonuria the feeding of the vitamin has no effect (537). An intermediate (?) in the oxidation of homogentisic acid, benzoquinone acetic acid, has also been detected in the urine of an ascorbic acid deficient patient (236). It leads to the production of methemoglobin.

The question therefore arises: is homogentisic acid a normal intermediate in the metabolism of the aromatic amino acids? If this be so, the substance should be readily oxidized in the normal animal. This has been found to be the case (225, 430). It also gives rise to acetoacetic acid (217) like phenylalanine and tyrosine.

The mode of formation of this phenol is not entirely clear. Early studies, reviewed by Neubauer (460), show that oxidation in the ring takes place before reduction in length of the side chain because no aromatic acid with two carbons in the side chain serves to raise the excretion of homogentisic acid. Presumably, oxidation also takes place before deamination because, as mentioned before, phenylpyruvic acid is not ketogenic like the parent amino acid (214, 463). Moreover, the initial oxidation takes place in the para position because neither ortho nor meta tyrosine increase homogentisic acid output (460). Schematically, the reactions dealt with may be shown as follows:



The conversion of the 4-hydroxy acid to the 2,5-dihydroxy acid presents a problem. It appears to be analogous to the following type reaction discovered by Bamberger (31, 378, 473), which takes place on oxidizing *p*-hydroxytoluene with Caro's reagent.



Isotopic experiments have confirmed the shift in the position of the side chain in the case of tyrosine (section VIII, 2).

In the one case of tyrosinosis so far described *p*-hydroxyphenylpyruvic acid was excreted even on fasting (439).¹⁶ When tyrosine was fed in relatively small doses (2–5 gm.) the following compounds also appeared in the urine: the substituted pyruvic acid increased,

¹⁶ It is perhaps noteworthy that the acid occurred in the urine in the enol form so that the urine gave no reaction for ketones, *e.g.*, no phenylhydrazine derivatives or bisulfite compound.

some tyrosine was excreted unchanged, and *p*-hydroxyphenyllactic acid appeared. With still larger doses of tyrosine, 3,4-dihydroxyphenylalanine also was found in small amounts. Fed phenylalanine was partly excreted as tyrosine and also in the forms previously mentioned. In this condition homogentisic acid is oxidized completely without apparent difficulty. From these data it is apparent that the patient lacked the ability to oxidize *p*-hydroxyphenylpyruvic acid to give homogentisic acid. The metabolic steps preceding and following this reaction went normally.

In premature infants deficient in ascorbic acid or in full term infants fed large doses of aromatic amino acids it has been found that tyrosine leads to the excretion of *p*-hydroxyphenyllactic acid along with smaller amounts of *p*-hydroxyphenylpyruvic acid (395). No homogentisic acid appears in the urine. It is rather surprising that in the infants the substituted lactic acid is excreted in larger amounts than the pyruvic acid because in both tyrosinosis and phenylketonuria the keto acid is the preponderant form in the urine. Generally, these abnormal products disappear from the urine when ascorbic acid is fed, and the ascorbic acid requirement appears to depend to some extent on the amount of aromatic amino acid in the diet. As might be anticipated, both D-ascorbic and D-isoascorbic acid are ineffective in this condition. Ascorbic acid only affects the metabolism of the L-forms of phenylalanine and tyrosine. It does not alter the formation of keto acid from the D-isomers of these amino acids.

Ascorbic acid deficient guinea pigs fed extra phenylalanine and tyrosine excrete homogentisic acid and *p*-hydroxyphenylpyruvic acid (536, 540), but the feeding of phenylpyruvic acid does not result in the appearance of abnormal metabolites in the urine (540). The abnormality of metabolism is also seen in slices of liver taken from the deficient animals (538). Such slices do not show an increase in oxygen consumption on addition of tyrosine unless ascorbic acid is added simultaneously. The increase in keto acid in the urine on feeding the D-isomers of phenylalanine and tyrosine is not affected by ascorbic acid (36). How the vitamin functions in this system is not clear although it is known to stabilize dopa (3,4-dihydroxyphenylalanine) while allowing its formation from tyrosine (509, 515). This may be due to the reduction of dopa quinone by ascorbic acid, or to the prevention of its formation.

There exists still another hereditary disease in which there is an anomalous form of phenylalanine metabolism associated with

mental deficiency. This is phenylketonuria which was discovered by Fölling and Closs (241) and investigated by others (481, 320). As the name indicates, fed phenylalanine is in part excreted as phenylpyruvic acid so that the amount of phenylpyruvic acid in the urine largely depends on the amount of the amino acid in the diet. However, part of the dietary phenylalanine is oxidized completely. The D-isomer of the amino acid is more effective in raising the ketone excretion than is the L. Tyrosine is without effect in this condition. Phenylpyruvic acid is oxidized with difficulty and phenyllactic acid is excreted as the ketone. The blood level of phenylalanine is high (322) and at the same time the urine excretion is increased (241), so it is concluded that the metabolic difficulty it concerned with the oxidation of phenylalanine to tyrosine. This is supported by results which show that in normal man an increase in hydroxyphenyl compounds in the blood follows administration of phenylalanine but not in the patients with phenylketonuria (321).

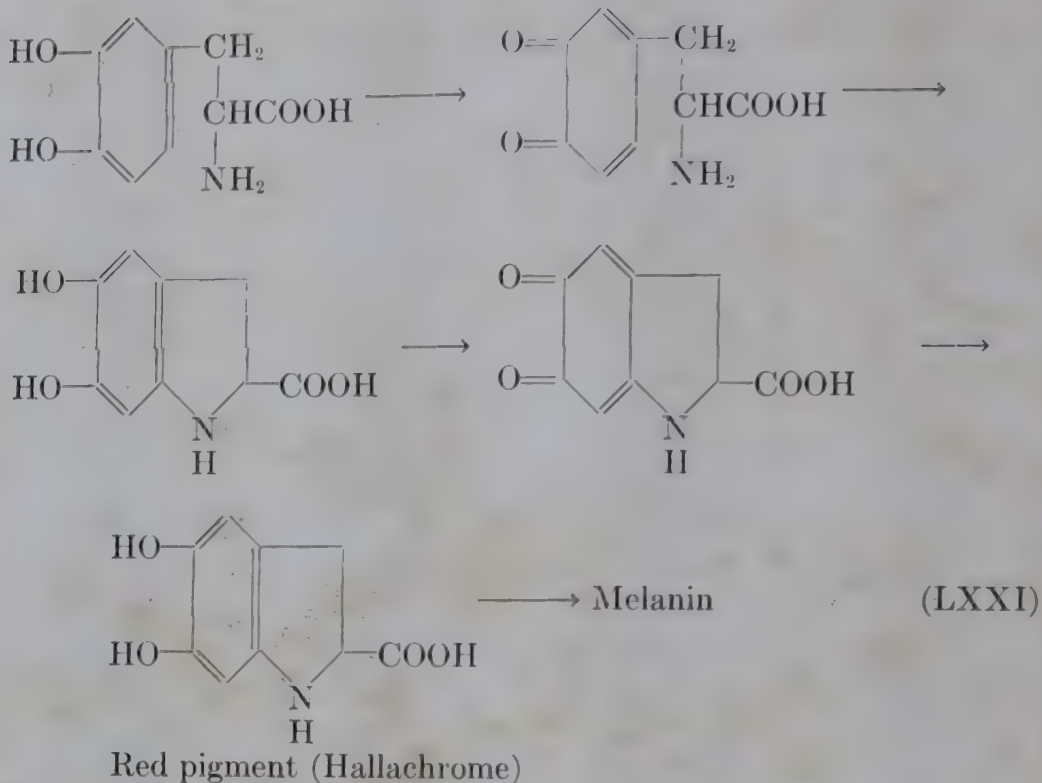
b. The 3,4-oxidation system

The existence of some other pathway of oxidation besides that through homogentisic acid is shown by the oxidation of *p*-methoxyphenylalanine and *p*-methoxyphenylalanine which proceed quite readily (180). Also, adrenaline is formed in some way by the oxidation of phenylalanine in the 3,4 position (280).



It is apparently formed from 3,4-dihydroxyphenylethylamine in the adrenal medulla (629). The occurrence of 3,4-dihydroxyphenylalanine in the urine of the patient with tyrosinosis has already been noted, so the 3,4-dihydroxyphenylethylamine is formed either by oxidation of phenylethylamine (187), tyramine (532), or by decarboxylation of dopa. *In vitro* it is possible to oxidize tyrosine to dopa (492) and tyramine to 3,4-dihydroxyphenylethylamine, and adrenaline-like substances are also produced by the action of tyrosinase (190, 293).

Melanin is produced from 3,4-dihydroxyphenylalanine by the action of dopa oxidase (306) and tyrosinase which are contained in the melanoblasts of the skin (77, 379). The reaction is pictured as follows by Raper (491, 234, 425):



In liver and kidney, 3,4-dihydroxyphenylalanine is metabolized by some other mechanism (309, 538).

Quite evidently thyroxine is formed from phenylalanine in the animal. In the test tube, diiodotyrosine in alkaline solution is converted in small yield to thyroxine (452, 323). A mechanism for the reaction has been proposed by Harington (290). The pathway *in vivo* has not been elucidated, although the iodination reaction can be demonstrated readily with radioactive iodine (446). Diiodotyrosine appears to be the biological intermediate in the synthesis of thyroxine (603).

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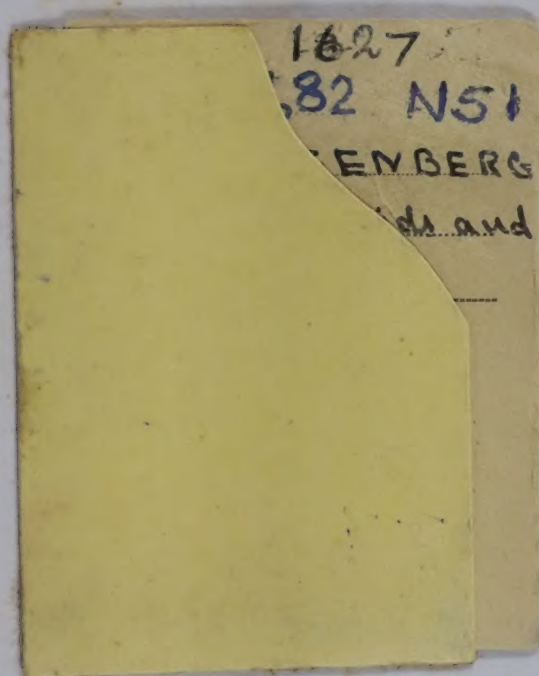
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